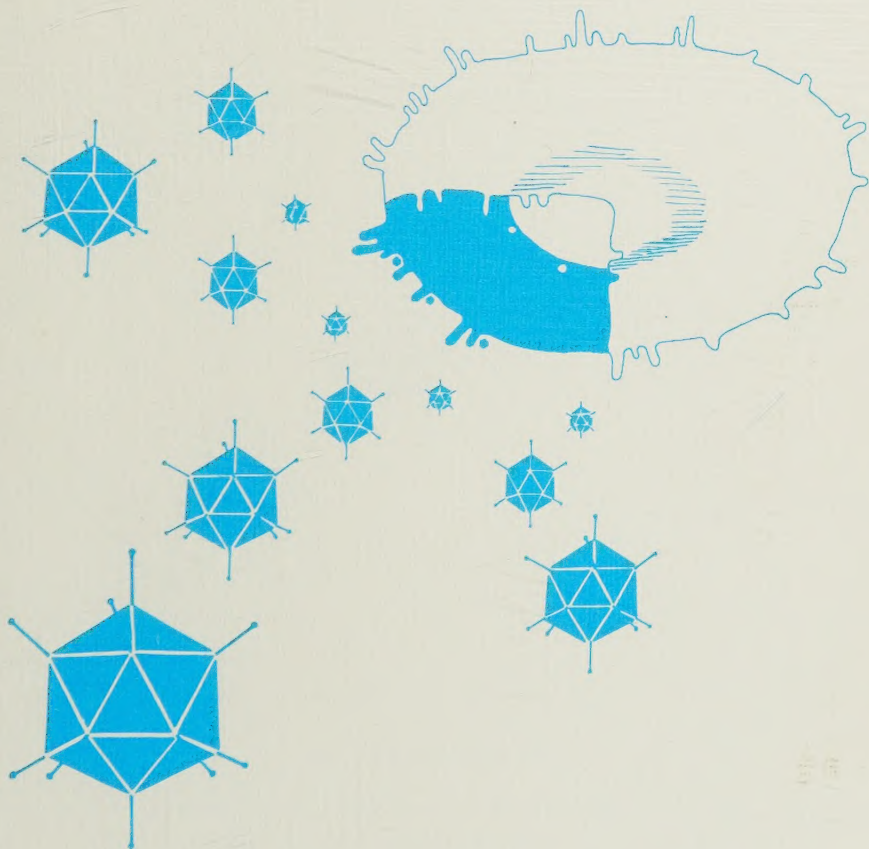


ENTRY OF ADENOVIRUS 2 INTO HeLa CELLS



Ulla Svensson



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Entry of adenovirus 2 into HeLa cells

av

Ulla Svensson

Fil.kand., Mlm.

AKADEMISK AVHANDLING

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Abstract The early events during adenovirus type 2 (Ad2) interaction with HeLa cells were investigated. Thus, a specific cellular receptor protein for Ad2 was isolated from the plasma membrane of such cells. It was identified as an integral membrane protein, containing N-acetyl-glucosamine residues, and displayed an estimated molecular weight around 40,000. Attachment of Ad2 at 37°C demonstrated a positive cooperativity, which could be inhibited by low temperature or glutaraldehyde fixation of the cells. The number of receptor sites per cell was around 5,000 at neutral pH. Uncoating of Ad2, rendering the genome sensitive to DNase treatment, started already at the outside of the plasma membrane. This process did not seem to be energy dependent. However, it was dependent on temperature, required an intact metabolizing cell, and bivalent cations - possibly linked to an enzymatic activity. Receptor-mediated endocytosis is suggested as one way of Ad2 entry into cells based on the following observations: a) The existence of a specific receptor, b) virions are rapidly internalized and can be seen inside coated pits and endosomes, c) the pattern of entry interference when various chemical reagents are used, and d) the temperature dependence of internalization. A proper destabilization of the virions on the plasma membrane and a low pH inside endosomes appear necessary for virions to subsequently leave the endocytotic vesicles. The penton base had a high affinity for the cell membrane at a pH around 5 and this interaction is suggested to mediate the transfer of virions from the acidic vesicles into the cytoplasmic compartment. The utilization of various antisera directed against virus structural proteins further strengthens the importance of different viral structural proteins and a correct conformational change during Ad2 entry and transfer to the cell nucleus.			
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ENTRY OF ADENOVIRUS 2 INTO HeLa CELLS

by

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Lund
Sweden

1985

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Cover:

..... and finally the goal was reached.

Ulla Svensson

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This thesis is based on the following communications, which will be referred to by their Roman numerals in the text.

- I. Svensson, U., R. Persson, and E. Everitt. 1981. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. *Journal of Virology* 38:70-81.
- II. Persson, R., U. Svensson, and E. Everitt. 1983. Virus-receptor interaction in the adenovirus system. II. Capping and cooperative binding of virions on HeLa cells. *Journal of Virology* 46:956-963.
- III. Svensson, U., and R. Persson. 1984. Entry of adenovirus 2 into HeLa cells. *Journal of Virology* 51:687-694.
- IV. Svensson, U. Significance of vesicles during adenovirus 2 internalization into HeLa cells. Submitted to *Journal of Virology*.
- V. Wohlfart, C., U. Svensson, and E. Everitt. Cellular distribution of Ad2 virions neutralized with different antisera. To be submitted.

INTRODUCTION

The research interest in the field of early interactions between animal viruses and cells has accelerated during the last ten years, both from a virological point of view but also as a model system for the entry of macromolecules into cells.

The early interaction between an animal virus and the cell is usually divided into three steps. The first being the attachment of virus particles to the cell surface. The second being the penetration of the virus inoculum from the outside of the cell into the cytoplasmic compartment. The third step contains the uncoating of the virion preparing its nucleic acid for subsequent gene expression and replication.

The attachment step

Virions may bind to lipids, carbohydrates or proteins on the plasma membrane of an animal cell. It is reasonable to assume that the structures which are recognized by the virus particles also have specific cellular functions besides being the virus attachment site. Despite this consideration the interaction between a virus and a structure on the cell surface can be specific and strong. Lonberg-Holm has suggested a specific terminology for virus attachment (51): Virus attachment protein(s) (VAP) are structures of the virion recognizing a cellular receptor; cellular receptor units (CRU) are cellular structures recognizing one VAP; cellular receptor sites (CRS) are cellular structures containing one or more CRU, which are engaged in binding a virus particle. In several virus systems the VAP structure has been identified, but the CRU or CRS are still poorly characterized in most virus systems.

A few reports have been published on the affinity between virions and pure lipids in liposomes or membranes (24, 73, 95). However, carbohydrate residues play an important role as virus binding sites for many viruses (29, 39, 104). Influenza virus A and B, paramyxovirus (29, 39, 104) and polyomavirus (30, 75) all have affinity for oligosaccharides with N-acetylneuraminic acid (NeuAc) in the terminal position and the oligosaccharide chain may be located either on a protein or on a lipid (7). For some viruses e.g. sendai virus, it is crucial that the NeuAc is bound to a glycoprotein (111). Ortho- and paramyxoviruses probably also use gangliosides as receptors (63, 100).

Protein components of the plasma membrane constitute the attachment structures for many viruses since treatment of cells with various proteolytic enzymes destroys the virion binding capacity of the cells (17, 55). Many proteins are glycosylated and it may be difficult to determine if it indeed is the protein or the carbohydrate moiety that primarily is responsible for the binding of virus particles. Treatment of cells with glycosidases or inhibitors of protein glycosidation therefore has emerged as a possibility to analyze whether it is the protein or the carbohydrate that serves as the attachment site. However, even when specific removal of the carbohydrate chain results in a decreased virus binding, it is possible that a conformational change in the protein moiety rather than the removal of the carbohydrate, causes the altered binding efficiency. The protein component of the CRS has been identified for a number of viruses (7, 17). Thus, the VAP of Semliki Forest virus (SFV) has affinity for the major histocompatibility antigenes (MHC) of human and murine cells (32). Despite this, Oldstone et al. (81) were able to infect two mouse embryonal carcinoma cell lines which were devoid of the MHC. This suggests that

the MHC most likely is not the only receptor for SFV. Indications that Epstein Barr virus (EBV) binds to the complement C'3 receptor are also available (112).

By saturation of the attachment sites on a cell with either picornavirus or with adenoviruses a partitioning of those viruses into receptor families has been performed (52). In this investigation it was found that adenovirus 2 shares receptors with coxsackie virus B3.

Virus induced changes have been observed in the plasma membrane at the stage of virus attachment to cells (47). Thus, it was shown that a variety of naked and enveloped viruses provoked a change in the membrane microviscosity, measured by the fluorescence polarization technique (49, 50). Lyles and Landsberger (56) have reported that crosslinking of receptors as a consequence of virus attachment and an involvement of microtubules are requirements for structural changes to occur in the plasma membrane.

The penetration step

Fusion and endocytosis are two well established ways of virus penetration through the plasma membrane (13, 55). A third mechanism, direct penetration, has been observed for a few viruses (17, 55). However, it seems that most viruses do not exclusively use one way of entry (7, 17). The further fate of a virion once inside a cell is dependent on that particular virus and on its location of replication (55).

Direct penetration: By this mechanism of entry, virions are internalized without being enclosed within vesicles or having undergone fusion with the plasma

membrane. Direct penetration has been described for poliovirus (20), adenovirus (4, 74), murine leukaemia virus (72), and reovirus (1). The events of this mechanism are unknown and most of the evidence for its existence comes from electron microscopic observations (4, 20, 72, 74). However, a few reports based on Cr-release analyses have also appeared (1, 99).

Endocytosis: Endocytotic uptake of virions was first observed by Fazekas de St. Groth in 1948, who named it viropexis (21). Entry by endocytosis may be nonspecific by invagination of noncoated regions of the plasma membrane, so called "bulk fluid endocytosis", or very specific by a mechanism called receptor mediated endocytosis (RME), defined by Goldstein et al. in 1979 (26). RME is a general mechanism for cells to take up various substances from the medium, for example hormones, asialoglycoproteins, lysosomal enzymes and low density lipoprotein (26). Virions of several systems have been shown to be internalized by this mechanism (22, 64, 65, 66). Because of small variations from the original definition of the RME the term adsorptive endocytosis has sometimes been used for virus uptake by a RME-like mechanism (64). Four criteria characterize the RME (26): 1) The binding component on the cell surface is a receptor in the strict sense, i.e. it is a molecule which function is to bind a ligand and thereby achieve a physiological effect; 2) Internalization of the ligand is effectively coupled to the binding event; the half-time for internalization is less than 10 min; 3) The receptor-bound proteins enter the cell through coated pits; 4) The internalized proteins are usually delivered to lysosomes where they are degraded into amino acids or they are further transferred into cellular structures other than the lysosome. RME starts with the ligand binding to cellular receptors which are clustered on the membrane or

which become clustered as a consequence of ligand binding. The clustering takes place inside coated pits (26). Some viruses always enter by coated vesicles while others may enter by uncoated vesicles (82). RME was first reported for reovirus, a member of the Reoviridae family (101). The vesicles containing internalized reovirus particles fuse with primary lysosomes. The passage of reovirus particles through lysosomes seems to be a prerequisite for a successful infection (101). One of the most thoroughly studied systems of virus-internalization is the RME of the Semlike Forest virus (31, 46, 64, 110). The low pH inside endosomes is a requirement for SFV to fuse with the vesicular membrane and thereby enter the cytoplasmic compartment (108). Endocytosis has also been reported as the entry mechanism of the two non-enveloped viruses polyoma virus and adenovirus (13, 22, 58, and below) and possibly also for picornavirus (8). For many viruses it appears that the endocytotic vesicle with its low pH, i.e. the endosome, forms the proper environment for virions to subsequently force the membrane barrier (64, 65, 82).

The uptake of ligands by endocytosis has been shown to be retarded at subphysiological temperatures (91, 107). Thus, a temperature dependence of internalization has been shown for the endocytotic uptake of SFV (64).

One method frequently used to study virus uptake has been by electron microscopy. Other approaches may be provided by the use of chemicals which for example inhibit various stages during the process of endocytosis. Lysosomotropic agents are applied to reveal the possible importance of a low pH inside an endocytotic vesicle (80). Model systems of virus-vesicle interaction have

also been constructed by the use of intact cells or liposomes (23, 53, 108, 110).

Fusion: A number of enveloped viruses can enter into cells by fusion of their membrane with the plasma membrane. Paramyxoviruses have become the type virus for such virus cell fusion studies (89). This process of entry has also been demonstrated for orthomyxovirus, vesicular stomatitis virus, togavirus (sindbis virus), herpesvirus and poxvirus (7, 17). For some viruses this mechanism seems to be the major way of entry, but other viruses may be internalized by RME as well (7, 17). Virus proteins responsible for the fusion event have been identified. Thus, the F-protein in the envelope of paramyxovirus and the HA₂-protein of orthomyxovirus are responsible for the fusing activity of those viruses (109). These proteins have to be proteolytically cleaved to become active and it is the hydrophobic nature of the cleaved proteins that mediates the fusion (109). Indications of a specific plasma membrane lipid involvement in the fusion process have also been described (46, 110). Another important factor during the fusion event is the pH (109), and members of the Paramyxoviridae family fuse at neutral pH, whereas viruses of the Orthomyxoviridae family require a pH between 5 and 6 (109). As a consequence of virus-cell fusion, alterations in the host cell plasma membrane have been described (77).

The uncoating step

Uncoating of most viruses is not a single event but at least a two step process which may occur at different compartments of the cell, such as at the plasma membrane, in an acidic vesicle or at the nuclear membrane. The extent of virus uncoating varies depending on the

mechanism of virus replication and there are indications that uncoating and intracellular transport are interdependent events. Thus, reovirus particles are modified inside lysosomes in such a way that penetration through a biological membrane will be possible (101). For enveloped viruses the removal of the envelope is the most important step during uncoating, since early macromolecular synthesis often can take place without a complete virion disassembly (55). When such viruses fuse with the plasma membrane, penetration and uncoating take place during the same event. Enveloped viruses, internalized by endocytosis, often fuse with the membrane of the endosome when the pH of the vesicle is decreased to a pH value between 5 and 6 (109). In these instances the true entry into the cytoplasm is accompanied by the step of uncoating. However, for many enveloped viruses additional uncoating takes place at other compartments of the cell (6).

Non-enveloped viruses may be uncoated by a mechanism which has been called "conformational uncoating" (17). This is the case for the picorna-, polyoma- and adenoviruses. For some picornaviruses the capsid changes its configuration during the attachment to the cell surface (15). For coxsackie- and poliovirus this process has been shown not to require energy or bivalent cations and may take place upon incubation of virions with isolated plasma membranes (28, 47). The configurational change of picornavirus makes the viral nucleic acid sensitive to nuclease treatment and this labilization of the capsid is probably of importance for the subsequent replication of the virus (62). The final stage of uncoating of picornaviruses probably takes place in the cytoplasm. Indications of a configurational alteration of the adenovirus capsid already at the outside of the plasma membrane have also been reported, see below. Papovaviruses which enter cells by RME have been shown to be transported to the cell

nucleus via vesicular structures and enter the nucleus in the form of distinctly recognizable virus particles as revealed in the electron microscope (58). Uncoating of these viruses takes place in the nucleus. However, it is still possible that these particles may have undergone some alteration at an earlier stage.

The model system

Human adenovirus perform a productive virus infection in a number of human cells. In this investigation HeLa cells - an established human cell line, originally obtained from a biopsy of a cervical carcinoma in 1951 (40) - was chosen for the investigation of the early events during a productive infectious cycle.

Adenovirus obtained its name because it was originally isolated from adenoid tissues. Since the first isolation of adenovirus in 1953 (37, 94) over 90 different serotypes have been isolated within the Adenoviridae family (78). These can be divided into mammalian and avian adenoviruses. Among the mammalian adenoviruses ca. 35 serological types of human adenovirus have been isolated (78). The human adenoviruses are divided into various subgroups depending on their ability to cause cancer in newborn mice and hamsters (41), or based on their ability to agglutinate red blood cells (93). The virus used in this investigation, adenovirus type 2 (Ad2), belongs to subgroup III according to hemagglutinating activity and this group corresponds to group C, which contains the non-oncogenic adenoviruses (41). However, like all human adenoviruses Ad2 has the capacity to transform non-permissive cells in cell culture (67, 68).

The adenovirus nucleic acid is surrounded by a protein capsid of icosahedral symmetry. The virus contains no lipids i.e. it is a non-enveloped virus. The virus particles have a diameter of 65-80 nm and the icosahedral capsid consists of 252 capsomers. The capsomers are held together by noncovalent bonds which can be artificially disrupted and the various intact capsomers can subsequently be isolated (25). Three proteins, the hexon, the penton and the fiber, are the dominant proteins of the virus capsid and constitute 60% of the total virion protein content (61). The hexon protein accounts for 240 of the 252 capsomers which form the twenty triangular facets of the capsid. The hexon protein is composed of three identical polypeptides with a molecular weight of 95,000-120,000 (12, 38, 60). The remaining twelve capsomers are the pentons, located in the twelve vertex regions of the icosahedron. The penton structure has an estimated molecular weight between 370,000 and 400,000 for Ad2 (85) and 500,000 for the type 5 penton (106). The penton consists of two subunits, the penton base and the fiber protein. The penton base of Ad2 has been suggested to consist of five or three identical polypeptides with a molecular weight of 70,000-85,000 (16, 61, 85). The fiber protein projects from the particle and is responsible for the attachment of adenovirus to cells (88). It consists of a polypeptide subunit of ca. 62,000 for Ad2 and Ad5 (19, 36, 43). The molecular weight of the native fiber protein of Ad2 has been estimated to 160,000-200,000 (16, 19, 102). These data indicate that the fiber is composed of three polypeptide subunits with similar molecular weight. However, recent results favouring a dimeric structure of the Ad2 fiber have been presented (16, 27). The Ad2 fiber is glycosylated and contains two N-acetylglucosamine residues per fiber molecule (43). The nucleic acid core, within the virions consists of a linear double stranded DNA of 35,937 nucleotides, which recently have

been sequenced (105). The nucleic acid is covalently linked to a 55.000 dalton protein (5, 92) and associated with a few virus specific basic proteins (5, 76).

PREVIOUS AND CONCURRENT INVESTIGATIONS

Attachment of adenovirus to cells

In the adenovirus system the fiber protein has been verified as the virus attachment protein because purified fiber antigen attached to cells efficiently blocks a subsequent virion attachment (88). Previous studies have indicated that plasma membrane components of protein nature and possibly glycosylated are important during the initial steps of virus-cell interactions (42, 69, 88). Hennache and Boulanger (33) have identified three KB cell plasma membrane polypeptides with affinity for the fiber protein of Ad2. Those polypeptides have an estimated molecular weight of 78,000, 42,000 and 34,000 (33). In another investigation Meager et al. (69) identified two glycosylated polypeptides with affinity for the Ad5 fiber. Despite the indications that the attachment site for adenovirus is glycosylated removal of NeuAc-residues from the cell surface, by neuraminidase treatment did not reduce the ability of cells to adsorb virions (3, U. Svensson and R. Persson, unpublished results); instead the attachment rate was initially somewhat increased. The number of cellular receptors have been estimated to 10^5 for the fiber protein and 10^4 for Ad2 or Ad5 particles (42, 88). This indicates that either a fraction of the binding sites is inaccessible to intact virions or the virus particles, by their twelve fiber projections, bind

multivalently to several receptor sites. During attachment of Ad2 at low temperature rearrangements of intramembranal particles and sterols have been observed in the plasma membrane of KB cells (34, 35). Patterson and Russell have shown that attachment of Ad5 to HeLa cells is followed by a temperature dependent redistribution of the virus particles on the cell surface (83). Inhibition of redistribution prevented a subsequent virus internalization.

Uncoating of adenovirus

By means of measuring the DNase sensitivity of the parental genome it has been shown that adenovirus particles are destabilized, in close connection to the penetration step of the plasma membrane (48, 86, 103). It is therefore possible that part of the vertex region of the capsid is removed by some mechanism accompanying the penetration event (57, 87). However, other investigations suggest that no proteins are lost from the virus particle during the penetration (71). Destabilization in vitro of Ad2 or Ad5 is not achieved in the presence of isolated lysosomal enzymes, suggesting that lysosomes are not involved in this event (2). In the electron microscope easily recognizable virions are transported to the nuclear pores (9). In some studies virions have been reported to lose their icosahedral symmetry and to become more rounded during the internalization process (4, 74). Despite the fact that electron microscopic observations show nearly unaltered virus particles in association with the nuclear pores, results from gradient centrifugations indicate that virions undergo a structural change during the transport to the cell nucleus (54, 71). Additional uncoating of adenovirus takes place near the nucleus where the virions, in an energy requiring process

(11, 74) "injects" the nucleic acid core into the nucleus. A biochemical analysis of the Ad5 infection revealed that the majority of the viral DNA enters the nucleus, whereas the viral protein is left on the outside (57). The final uncoating takes place within the nucleus and the end product of this process is probably viral DNA covalently linked to a viral protein (92). Lonberg-Holm and Philipson (54, 55) divide the uncoating of adenovirus into the following three steps: 1) Soon after virus attachment to the cell surface a DNase sensitive particle is produced. This particle is recovered in the cytoplasm and the penton-fiber is partly or totally missing. 2) The DNA-protein core is removed from the hexon-capsid at the nuclear membrane. 3) The final uncoating takes place during the second hour of infection, in the nucleus of the infected cell. The mechanism of core protein removal from the DNA is unknown.

Adenovirus penetration

Adenovirus have been reported to force the plasma membrane by endocytosis (9, 22) or by direct penetration (4, 74). The conclusion that a mechanism of direct penetration exists comes from transmission electron microscopic studies and by the freeze etching technique (4, 74). Since virions can be seen in coated or uncoated vesicles in the cytoplasm (13, 83), the mechanism of adenovirus entry which has gained most support is the one by endocytosis (13, 22, 98). Some virus types, e.g. Ad2 and Ad5, seem to leave the vesicles rapidly whereas for example virions of Ad7 appear to be transported to a lysosomal structure, where they still are recognizable after six hours (10, 79). The importance of acidic vesicles during Ad2 internalization has indirectly been shown by using a *Pseudomonas* toxin conjugated to epidermal

growth factor (98). The presence of adenovirus enhances the toxicity of the toxin, probably because the virus enters the cells via the same vesicular structures as the toxin and further by disrupting these vesicles (22). The importance of a low pH inside those vesicles and the involvement of the adenovirus penton base protein was demonstrated by using lysosomotropic agents and antibodies directed against the penton base (97, 98). Adenovirions which have succeeded in becoming free inside the cytoplasmic compartment have been seen in association with microtubular structures during the further transportation to the cell nucleus (14, 70).

THE PRESENT INVESTIGATION

The intention with the present investigation was to elucidate some of the events behind the mechanism of Ad2 entry into HeLa cells.

The event of adenovirus attachment (I, II)

Plasma membranes from HeLa cells were isolated by a two-phase polymer system. Various methods to solubilize the CRS for Ad2 from these membranes were compared and the different degrees of solubilization indicate that the CRS is an internal membrane protein. The solubilized membrane material was analyzed for receptor activity in a standardized virus attachment inhibition assay. The most efficient way of solubilizing such a receptor activity was by using 0.5% Triton X-100 and consequently this method was chosen for all further investigations. The

solubilized receptor active material was relatively stable and could be stored at 4°C for one week or at -20°C for at least seven weeks without loss of activity (U. Svensson, unpublished data). This material contained a polypeptide of 40,000 dalton which had high affinity for adenovirus as revealed by sucrose gradient analyses. To further characterize the proteins that interfered with Ad2 attachment, affinity chromatography on a wheat germ agglutinin (WGA) and on an Ad2-AH-Sepharose column was performed. The fraction with high affinity for WGA was enriched in polypeptides with affinity also for Ad2 on the AH-Sepharose column. This suggests that the CRS for adenovirus is a glycoprotein containing N-acetylglucosamine residues. Two polypeptides with molecular weight of 42,000 and 40,000 dalton were predominant. This material was analyzed by the virus attachment inhibition assay, but curiously enough it did not inhibit the virus attachment. However, later studies (IV) have shown that the receptor for the fiber protein is irreversibly destroyed at low pH and a pH of 2.6 was used during the elution of material from the Ad2-AH-Sepharose column.

In paper number II the influence of temperature on the Ad2 attachment was analyzed. Thus, an inflection point for the attachment process was obtained at ca. 20°C. The inflection point was absent in cells treated with 0.015% glutaraldehyde. The multiplicity dependence of virus attachment was also studied, and when these results were transferred into Scatchard plots a positive cooperative binding of the virions was obvious at 37°C. If the experiment was performed at 3°C or with glutaraldehyde fixed cells no positive cooperativity was found. Positive cooperative binding is common in systems where a multivalent ligand attaches to a receptor (45, 90). The requirements for such a binding in the Ad2 system appear to be a multivalent ligand and the possibility of lateral

diffusion of the CRU in the plasma membrane (II, 84). Thus, the positive cooperativity can possibly represent a patching of many CRU into CRS. Indication that this patching - cooperativity preceeds the destabilization of virus particles attached to the plasma membrane have been obtained (III, 84). From the Scatchard plots the number of CRS for Ad2 on HeLa cell was estimated to around 5,000.

Direct observation of virions attached to the cell surface was performed by rhodamine labeling of the virions and by employment of the fluorescence microscope. Capping of a fraction (ca. 15%) of the rhodamine labeled virions could be seen when the cells were incubated at 37°C. Capping was inhibited by low temperature, sodium azide, cytochalasin B and 2-deoxyglucose, but not by colchicine. This pattern corresponds well with the capping phenomenon when immunoglobuling or lectins attach to cells (96). The relevance of capping for an Ad2 infection is difficult to interpret with the knowledge of today.

Destabilization of the virions, penetration and transport to the cell nucleus (III, IV, V).

It has previously been shown that Ad2 are destabilized, rendering the virus genome sensitive to DNase treatment, in close connection to the internalization event (48, 86, 103). This destabilization showed a strong temperature dependence (III). An Arrhenius plot of the initial rate of uncoating demonstrated an inflection point at 16°C. Activation energies of 331 kJ/mol below and 88 kJ/mol above this temperature correspond to membrane processes below and above a lipid phase transition (44) and established systems of endocytosis (59). Also for the pene-

tration step a strong temperature dependence could be observed; thus, below 15°C no virions entered into the cells but at 17°C a few per cent of the virions had succeeded in becoming free in the cytoplasm. To obtain more information about the mechanism of Ad2 entry, the influence of various chemicals on the steps of virus uncoating, penetration, and transport to the cell nucleus was analyzed by biochemical techniques. In addition virus specific early and late polypeptide production was measured when the reagents had been present during the first hour of incubation. Those studies were supplemented with an electron microscopic investigation on the location of virions in the presence of the same reagents. Penetration of Ad2 through the plasma membrane was completely inhibited by sodium azide, whereas uncoating was only slightly influenced. This indicates that uncoating takes place already at the outside of the plasma membrane. When Ad2 was incubated with isolated plasma membranes, cell homogenates or glutaraldehyde fixed cells no destabilization occurred, demonstrating that intact and metabolizing cells are required for the uncoating process. Based on the inhibitory patterns of EDTA, EGTA, dansylcadaverine and DTT, it is suggested that the uncoating process of virions is dependent on enzymatic activities and follows upon a reorganization in the plasma membrane. When the situation in the presence of DTT was further analyzed by electron microscopy, 70% of the virions were left on the cell surface or inside multivesicular bodies (MVB) or lysosomes. This is the same fraction of virions which did not become DNase sensitive at the cell surface. When glutaraldehyde fixed virions were used it was even more obvious that virions not being sensitive to the destabilization process were accumulated in intracellular vesicles. Thus, an early labilization of the virions was a prerequisite for an escape from the endosome.

In control cells around 70% of the cell attached virions had become free in the cytoplasm. Indications of the involvement of microtubuli in the further transfer of virions to the cell nucleus was obtained when colchicine was present in the electron microscopic investigation. However, when a later viral gene expression was measured, after colchicine had been present during the first hour of infection, neither an involvement of microtubuli nor microfilaments could be seen. The major route for Ad2 entry appears to be the RME, which means that the virions have to escape from intracellular vesicles before reaching the cell nucleus. The utilization of lysosomotropic agents when studying the Ad2 transport to the nucleus or the synthesis of an early viral polypeptide indicated the importance of an acidic milieu inside the endocytotic vesicle for a successful infection. However, the inhibition of nuclear transport was never more than 70%. An inverted model system of the endocytotic vesicle was constructed by incubating Ad2 particles or different virus structural proteins together with intact cells in buffers of different pH. It was shown that attachment of Ad2 have two pH optima, one at pH 5.0 and another at pH 6.5. At pH 5.0 the attachment seems to be due to the penton base and at pH 6.5 to the fiber. The number of receptor sites per cell was 25,000 and 6,000 at low and neutral pH, respectively. These results suggest that the structure (s) recognized by the virus at low pH is not the same as under neutral pH conditions and possibly the affinity between the penton base and the membrane is important inside the endosomes when the virus particles enter into the cytoplasmic compartment. The fiber protein, attached to cells under physiological conditions is easily detached at a pH below 5.2 and a preincubation of cells at an acidic pH leads to an irreversible denaturation of the receptor site for the fiber protein. Ligand-receptor dissociations at low pH are well established phenomena in

systems of RME (18). However, release of complete virions after attachment seems to be difficult, because neither a change in pH nor the utilization of proteases caused a dissociation of Ad2 from the cells (IV, U. Svensson, unpublished results).

Antibodies directed against various viral structural proteins were used to collect more data on the mechanism of Ad2 entry into cells. It was shown that virions which had reacted with antifiber-antibodies, at a serum dilution that completely neutralized a virus infection, still had the capacity to attach to the cell surface; however, such virus had a reduced capacity to become DNase sensitive and they were almost quantitatively unable to enter into the cells. The few virions that had entered were located as large aggregates inside vesicles, probably lysosomes. After incubation with anti-hexon-serum, also at concentrations that completely neutralized the virus infection, the majority of the virions entered the cells but could not leave the endocytotic vesicles. Anti-penton base-serum was not able to neutralize Ad2 to an extent more than 50%, even if high concentrations were used, and this serum allowed half as much of the virions to reach the nucleus as in control cells. The majority of the virions which did not reach the nucleus were trapped inside vesicles, possibly because the penton base interaction with the endosome membrane was blocked. The fact that anti-penton base-antibodies never inhibited the Ad2 infection to an extent greater than 50% and that the lysosomotropic agents did not inhibit virion transport to the nucleus by more than 70%, suggests the existence of alternative ways for the Ad2 internalization. One is the RME requiring a low pH inside endosomes with the concomitant interaction between the penton base and the endosome membrane enabling Ad2 to enter into the cytoplasmic compartment. The other entry mechanism is at

present unknown, but it cannot be excluded that a fraction of the virions are internalized by the previously described (4, 74) mechanism of direct penetration.

CONCLUDING REMARKS

From the results in this investigation I finally would like to make a suggestion about the steps included during the entry of Ad2 into cells.

- Ad2 attaches to a specific CRS on the cell surface. The CRS is of protein nature, contains N-acetyl-glucosamine residues and the predominant structure has a molecular weight of 40,000 dalton.
- A cooperative binding of virions at 37°C requires a multivalent ligand and the possibility of lateral diffusion of membrane components.
- The uncoating of Ad2 starts already at the outside of the plasma membrane where virions become sensitive to DNase treatment. This process is not energy dependent.
- The destabilization process is temperature dependent, requiring an intact metabolizing cell and bivalent cations possibly linked to an enzymatic activity, e.g. a transglutaminase.
- The conditions for a positive cooperative binding and the destabilization process are similar, suggesting that those processes are closely connected.

- The existence of a specific receptor, the fact that virions rapidly are internalized and can be seen inside coated pits and endosomes, the pattern of interference when various chemicals are used, and the temperature dependence of internalization all are factors suggesting that RME is one way of Ad2 entry into cells.
- A proper destabilization of the virions on the outside of the plasma membrane appears necessary for virus to be able to leave the endocytotic vesicles.
- A low pH inside endosomes was shown to be important for the internalization of at least a fraction of the virions.
- Interaction between the penton base and the membrane of the acidic vesicles mediates the internalization of virions into the cytoplasmic compartment.
- The importance of the various viral structural proteins and the requirements of correct conformational changes during adenovirus entry and transfer to the cell nucleus were further strengthened by the fact that virions incubated with antisera directed against various structural proteins never reached the nucleus.
- The employment of anti-penton base-antibodies and lysosomotropic agents suggest the involvement of an alternative mechanism for Ad2 internalization besides the RME.

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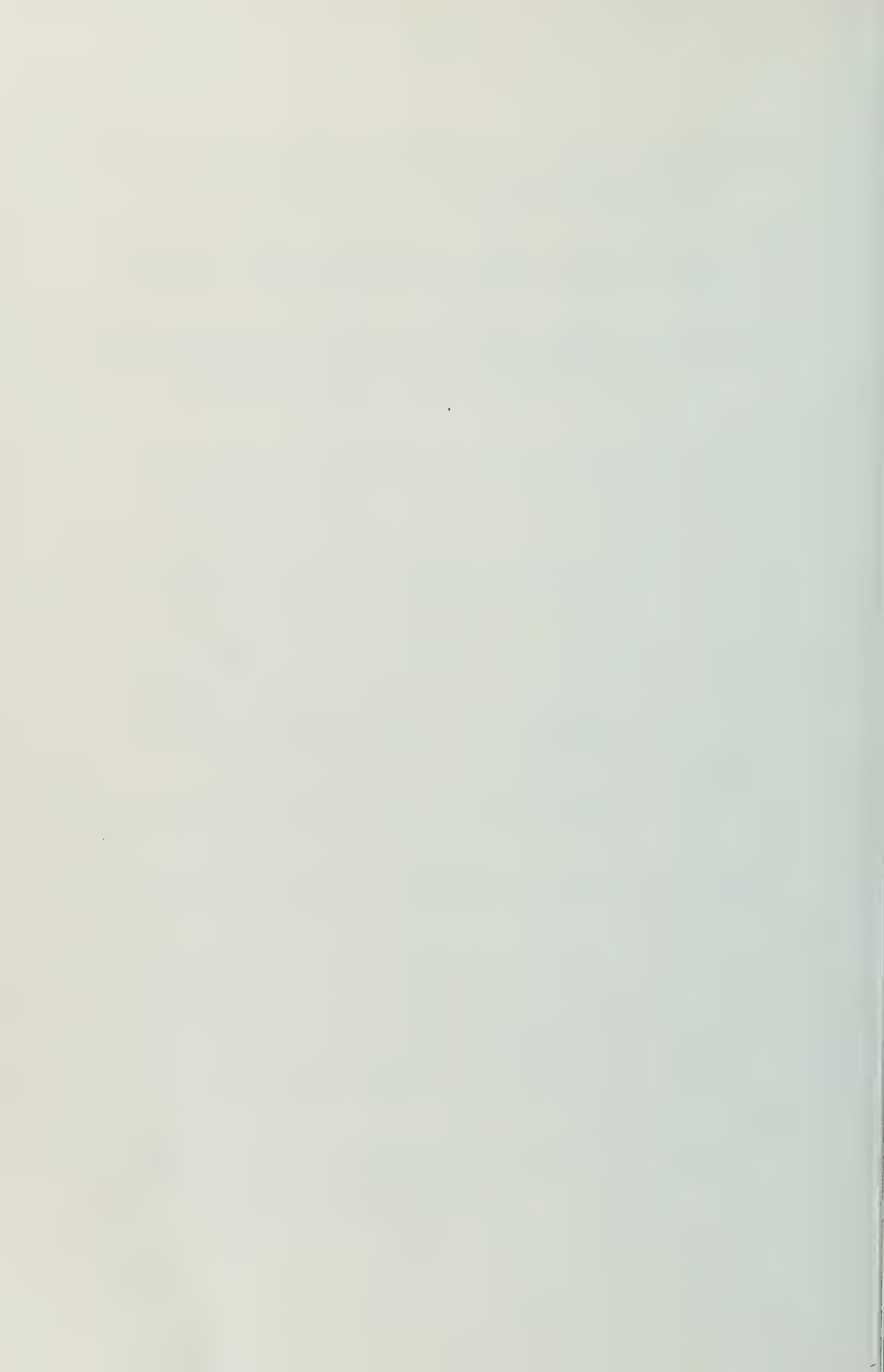
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Virus-Receptor Interaction in the Adenovirus System

I. Identification of Virion Attachment Proteins of the HeLa Cell Plasma Membrane

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Plasma membranes from HeLa cells were isolated in a two-phase polymer system. To compare the efficiency of attachment protein extraction, a normalized assay for the assessment of adenovirus type 2 (Ad2) receptor-active components interfering with the attachment of Ad2 to HeLa cells was developed. An optimized detergent extraction procedure, 0.5% Triton X-100, was used, and solubilized membrane proteins were radioisotope labeled *in vitro*. Proteins with affinity for Ad2 virions were quantified and identified in a sucrose gradient sedimentation assay and by affinity chromatography with cross-linked Ad2 virions immobilized to AH-Sepharose 4B. From virions recovered in the sucrose gradient system, one major membrane component of high affinity was identified with a polypeptide molecular weight of around 40,000. Glycosylated proteins isolated by wheat germ lectin chromatography with high affinity for immobilized virus particles were isolated, and two major components with apparent molecular weights of 40,000 and 42,000 were identified. We suggest that a glycosylated protein with high affinity for Ad2 virions and a polypeptide molecular weight of 40,000 to 42,000 is one component of the Ad2 attachment site on HeLa cells.

All virus infections start with the viruses recognizing components on the surface of the cell that is about to be infected. The extracellular interactions between animal viruses and cells have been extensively reviewed and described by Lonberg-Holm and Philipson (27). More recently, Kohn has elaborated on a selected number of viruses interacting with cellular membranes (24).

The cellular surface components of interest for the infecting virions have usually been referred to as viral receptors. A more appropriate term, virus attachment proteins, has been suggested (32); this term implies that the cells do not synthesize membrane components for the *a priori* function of being the structures responsible for reception of future viral infections.

In the adenovirus system, the virion part of the virus-cell-recognizing system is the well-characterized fiber antigen (10, 34, 37) located at the 12 vertices of the virion (38). Previous studies also indicate that plasma membrane components that are protein in nature, and possibly glycosylated, are important in the initial steps of virus-cell interaction (31, 34). Attempts have been made to isolate and characterize the components of the attachment site for adenovirus type 2 (Ad2) on KB cells, using highly purified fiber and penton structures. Hennache and Boulanger (18) identified three major Ad2-fiber-rec-

ognizing polypeptides with apparent molecular weights of 78,000, 42,000, and 34,000.

With an assay designed to measure and normalize adenovirus attachment protein activity of HeLa cell plasma membrane extracts, we tried to optimize the conditions for preparation of virus recognition proteins. Throughout efforts to identify and isolate these components, *i.e.*, by sucrose gradient sedimentation analyses and affinity chromatography techniques, complete virus particles were used to mimic the complex situation *in vivo*.

MATERIALS AND METHODS

Cells. HeLa cells were maintained in suspension cultures at cell densities of 2×10^5 to 6×10^5 cells/ml in Eagle minimal essential medium (EMEM) supplemented with 8% fetal calf serum and gentamicin (5 μ g/ml).

Infections and virus purification. HeLa cells grown in spinner cultures were synchronously infected with Ad2 at a multiplicity of 2,000 particles per cell as previously described (13). For radioisotope labeling, virus was propagated in the same way. [3 H]thymidine (56 Ci/mmol; 25 μ Ci/ml) was added 10 h postinfection and maintained until harvest of cells at around 40 h postinfection. Virus was purified and stored as described elsewhere (12).

Plasma membrane isolation. HeLa cell plasma membranes were isolated from batches of 5×10^8 cells in a discontinuous sucrose gradient system as described by Bosmann et al. (5), modified with a cushion

of 60% (wt/vol) sucrose applied at the bottom of the centrifuge tubes. Five fractions were collected from each tube and denoted as follows: fraction I, the cushion of 60% sucrose; fraction II, the density region corresponding to the interphase between the sucrose concentrations of 45 and 35%, including the entire portion of 45% sucrose; fraction III, the interphase region between the sucrose concentrations of 35 and 25%; fraction IV, the interphase region between concentrations of 25 and 20%; and fraction V, the remaining uppermost portion.

Plasma membranes were also prepared in a two-phase polymer system consisting of polyethylene glycol 6000 and dextran T-500 as described by Brunette and Till (7) and modified by Jay et al. (22). Batches up to 2×10^9 cells were processed, and 5×10^8 cells per 120 ml of mixed-phase system were used. At the end of the separation, the interphase was collected and diluted five times in 50 mM Tris-hydrochloride buffer, pH 7.3. The membranes were sedimented at $4,500 \times g$ for 15 min at 4°C , washed once, and resuspended in the buffer above to yield a protein concentration of 4 to 8 mg/ml.

Enzyme marker assays. 5'-Nucleotidase was assayed by the method of Heppel and Hilmoe (19) as modified by Johnsen et al. (23). Na^+ , K^+ -ATPase was determined by the method of Wallach and Kamat (39). Phosphate determinations were in both cases done according to the method of Chen et al. (9). Reduced nicotinamide adenine dinucleotide phosphate cytochrome *c* reductase was assayed by the method of Hrycay and O'Brien (20). Protein was determined by the method of Hartree (16), with bovine serum albumin (BSA) as the standard.

Solubilization of membrane proteins. All solubilization experiments were carried out on plasma membranes isolated by the two-phase method.

(i) High-salt procedure. Solid KCl (36) was added to a final concentration of 3 M; the samples were then shaken until the salt dissolved and further incubated for 16 to 20 h at 8°C . The samples were subsequently centrifuged at $100,000 \times g$ for 1 h at 4°C , and the supernatants were dialyzed against 50 mM Tris-hydrochloride buffer, pH 7.3.

(ii) Organic solvent procedures. Butanol (29), pentanol (40), and pyridine (3) were separately applied as extracting agents, each at a final concentration of 10% (vol/vol). After incubation for 1 h at 8°C with shaking every 15 min, the samples were centrifuged and dialyzed as described for the high-salt procedure.

(iii) Detergent procedures. Various concentrations of the following detergents were used: Triton X-100 [polyoxyethylene(9,10)-*p*-*t*-octyl-phenol], Tween 20 (polyoxyethylene sorbitan monolaurate), and sodium deoxycholate (DOC) (17). After detergent treatment, the samples were incubated for 1 h at 8°C with shaking every 15 min and finally centrifuged as above. The dialysis step was omitted.

In vitro labeling of proteins. Triton X-100-solubilized proteins were labeled for 2 h at 26°C by reductive alkylation, using sodium cyanoborohydride and [^3H]formaldehyde as described by Dottavio-Martin and Ravel (11). The reaction mixtures were passed through PD-10 columns with Sephadex G-25M equilibrated and eluted with 40 mM sodium phosphate buffer, pH 7.0, containing 0.037% Triton X-100. The

labeled proteins were recovered in the void volume and monitored by assessment of trichloroacetic acid-precipitable radioactivity. The specific radioactivity of labeled proteins ranged from 2.7×10^7 to 3.0×10^7 cpm per mg of protein.

Labeling of membrane proteins with the Bolton-Hunter reagent was performed essentially as described by the manufacturer. After labeling, the samples were separated from unincorporated isotope by passing through a PD-10 column with 0.15 M NaCl-0.037% Triton X-100 in 0.015 M sodium phosphate buffer, pH 7.3, as the eluant. Labeled material was detected by radioactivity measurements without prior precipitation. Labeling efficiencies were in the range of 90%, and the specific activities of labeled material were about 9×10^6 cpm/mg of protein.

Assay of receptor-active material. The principle of the assay was based on the assumption that solubilized plasma membrane proteins compete with viruses during the course of virus-cell interaction and thus inhibit virus attachment to cells.

(i) Incubation 1. Five microliters of highly purified [^3H]thymidine-labeled Ad2 virions (8.5×10^9 particles; 69,400 cpm) was mixed with 120 μl of double-distilled water (for the control series) or with 120 μl of protein sample (for the sample series) and 125 μl of $2 \times$ EMEM designed for suspension cultures without serum, which was omitted in all subsequent steps. To ensure proper solubility of all reagents, Triton X-100 was added at a final concentration of 0.037% to all incubation mixtures. When extracts containing Tween 20 or DOC were analyzed, the final concentrations of these detergents also were adjusted to 0.037%. This concentration of these detergents has previously been shown not to affect virus attachment to HeLa cells. The mixtures were incubated for 1 h at 37°C on a shaking water bath and then centrifuged at $140 \times g$ for 5 min at room temperature. The supernatants were transferred to round-bottom plastic tubes, and 2.5×10^7 previously EMEM-washed HeLa cells were added in a volume of 250 μl . This gave a final concentration of 5×10^7 cells/ml and a multiplicity of infection of about 340 particles/cell.

(ii) Incubation 2. The cell-containing mixtures were incubated for 1 h at 37°C as above, and samples were withdrawn at different intervals, diluted 10 times in EMEM, and centrifuged at $140 \times g$ for 3 min. The radioactivity in the supernatants and the pellets was measured and used for calculating the degree of virus attachment. Three-point attachment curves were always constructed for the samples analyzed.

(iii) Calculations. The percentages of radioactivity remaining in the supernatants after 60 min of attachment were used to calculate the relative inhibition of virus attachment. This procedure was believed to counteract any possible variations in the status of the cells. Calculations were performed according to the formula:

relative inhibition

$$= \frac{\begin{array}{l} \% \text{ cpm in supernatant (sample)} \\ - \% \text{ cpm in supernatant (control)} \end{array}}{\begin{array}{l} \% \text{ cpm in supernatant} \\ \text{(maximum inhibition)} \\ - \% \text{ cpm in supernatant (control)} \end{array}} \times 100$$

where percentage of counts per minute in supernatant (sample) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the presence of presumptive receptor material; percentage of counts per minute in supernatant (control) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the absence of interfering material; and percentage of counts per minute in supernatant (maximum inhibition) is the radioactivity remaining in the supernatant at maximum inhibition of attachment in the presence of, for example, detergent-extracted plasma membrane proteins. The highest measured inhibition was 95% after 60 min of virus-cell interaction. The relative inhibition revealed by different quantities of Triton X-100 (0.5%)-extracted proteins was determined, and a dose-response curve was constructed (see Fig. 3 in Results). We assumed that dose-response curves of similar shape would be obtained with proteins of other extraction procedures. This would enable us to compare the efficiency of virus attachment inhibition of other protein extracts. The degree of inhibition of all extracts was normalized to a 50% relative inhibition of attachment in the Triton X-100 system, using 0.5% detergent at extraction.

Rate zonal sedimentation analysis of virus-attached plasma membrane proteins. Highly purified Ad2 virions were mixed with solubilized plasma membrane proteins radioactively labeled *in vitro*. The amount of virus and protein used corresponded to multiplicities of infection between 6,000 and 8,000 particles per cell equivalent based on calculations of extraction efficiencies and original cell quantities used for plasma membrane preparations. The samples were incubated in the presence of 0.037% Triton X-100, 0.5% Triton X-100, 0.2 M NaCl, 1% BSA, or 3% BSA for 1 h at 37°C with shaking. Except for the incubation with 0.5% Triton X-100, all incubation mixtures were supplemented with Triton X-100 to give a final concentration of 0.037%. The mixtures were subsequently layered onto linear sucrose gradients consisting of 2 × 1.6 ml of 20 to 40% (wt/vol) sucrose in 50 mM Tris-hydrochloride buffer, pH 7.5, and containing the same amount of detergent, salt, or BSA as the pertinent incubation mixture. The gradients were underlaid with 0.2 ml of 60% (wt/vol) sucrose in the same buffer system and centrifuged at 100,000 × *g* for 40 min at 6°C. After fractionation into 25 fractions by puncturing from the bottom, radioactivity was monitored by counting 5-μl portions. Fractions of interest were pooled, dialyzed against double-distilled water, freeze-dried, and analyzed electrophoretically on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.

Chemical cross-linking of Ad2 virions. Glutaraldehyde was used to stabilize Ad2 by cross-linking in the presence of 0.1 M sodium phosphate buffer, pH 7.4, in a procedure described by Avrameas and Ternyck (2) with some modifications. The concentrations of glutaraldehyde and virions were determined so as to maintain the virus particles in a true soluble state as well as provide the highest coupling efficiency of virions to AH-Sepharose 4B. In the procedure chosen, glutaraldehyde (25% solution) was added to a 5 optical density units solution of Ad2 in sodium phosphate buffer to make the concentrations of glutaraldehyde and sodium phosphate 5% and 0.1 M, respectively.

The total 3-ml volume of the mixture was incubated for 24 h at 8°C, whereafter it was freed of glutaraldehyde by filtering through a PD-10 column with Sephadex G-25M in 0.1 M sodium phosphate buffer, pH 7.4. Fractions were pooled after measuring the absorption at 260 nm. The stabilized Ad2 virions were further immobilized and used as an affinity adsorbent (see below).

Fractionation of material with Ad2 virion affinity from the HeLa cell plasma membrane. (i) Wheat germ lectin-Sepharose 6MB chromatography of solubilized membrane proteins. A column with 5 ml of wheat germ lectin-Sepharose 6MB was equilibrated with WGA buffer (0.2 M NaCl and 0.037% Triton X-100 in 0.05 M sodium phosphate buffer, pH 7.0). [³H]formaldehyde-labeled, Triton X-100 (0.5%)-solubilized membrane proteins were applied to the column after dialysis against WGA buffer for 18 to 20 h at 4°C. Fractions of 0.5 ml were collected at a flow rate of 3.4 ml/h. After the column was washed with WGA buffer, the retained proteins were eluted with *N*-acetyl-D-glucosamine (100 mg/ml) in WGA buffer. The elution profile was followed by direct counting of 10-μl portions from each fraction. The peak fractions of interest were pooled and dialyzed against WGA buffer for 20 h at 4°C.

(ii) Affinity chromatography on an immobilized adenovirion matrix. Highly purified Ad2 virions were stabilized by cross-linking as described above and incubated with carefully washed AH-Sepharose 4B (up to 2.5 optical density units of Ad2 per ml of gel) for 24 h at 4°C in a rolling state. Ethanolamine was added (1 M, final concentration) to block any residual aldehyde groups, and the mixture was incubated for another 12 to 15 h at 4°C. After the second incubation, the gel was washed extensively with WGA buffer and was then ready to use. By using radioactively labeled Ad2 virions, it was established that the coupling efficiency to AH-Sepharose 4B was 90 to 95%. Moreover, when coupled viruses were treated with buffer solutions at low pH (0.2 M glycine-hydrochloride, pH 2.2), at high pH (0.2 M glycine-NaOH, pH 12.0), at high ionic strength (5 M MgCl₂ in 50 mM Tris-hydrochloride, pH 7.4), and containing detergent (1% Triton X-100 in 50 mM Tris-hydrochloride, pH 7.4), no loss of virus was detected. Therefore, it was assumed that the viruses were covalently linked to the matrix.

Immobilized virions on an Ad2-AH-Sepharose 4B column were able to adsorb antifiber antibodies, indicating the presence of immunologically intact and accessible fibers of the virions. This immunological procedure was used to monitor the stability of the columns used throughout these investigations.

Unretained and retained fractions from the lectin chromatography step (see above) were analyzed on the Ad2-AH-Sepharose 4B (1.2 optical density units of Ad2 per ml of gel), equilibrated with WGA buffer. The pertinent fractions were applied to the column, and 3.2-ml fractions were collected at a flow rate of 3.4 ml/h. After washing of the column with WGA buffer, proteins of low virus affinity were eluted with WGA buffer containing 0.5 M NaCl. Proteins with high affinity for Ad2 virions were subsequently eluted with 0.1 M NaCl-0.037% Triton X-100 in a 0.1 M glycine-

hydrochloride buffer, pH 2.6. Fractions obtained from elution at low pH were immediately neutralized with 1 M sodium phosphate buffer, pH 7.2. The elution profiles were monitored by direct radioactivity measurements of 50- μ l portions withdrawn from each fraction. Fractions constituting the peaks of interest were pooled, dialyzed against double-distilled water, and freeze-dried.

SDS-PAGE. The SDS-polyacrylamide gel electrophoresis (PAGE) analyses were performed on gel slabs (1.5 by 70 by 70 mm) with 13% acrylamide and 0.35% bisacrylamide essentially as described by Maizel (30). Fluorography was done according to Bonner and Laskey (4).

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were done in a cocktail of toluene-methanol (1:1) with 0.4% Omnifluor. Assessment of trichloroacetic acid-precipitable radioactivity was done on filter paper disks (Munktell, Grycksbo, Sweden, OOH; 21-mm diameter). Filters with added and dried samples were submerged into 10% ice-cold trichloroacetic acid for at least 30 min and then washed twice in 5% cold trichloroacetic acid and twice in 70% cold ethanol. The dried filters were counted in toluene with 0.4% Omnifluor. The 125 I radioactivity was measured in the tritium channel with a 60% efficiency. A Nuclear-Chicago Mark II liquid scintillation counter was used to determine radioactivity.

Chemicals and radioisotopes. EMEM, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. [3 H]-formaldehyde (0.1 Ci/mmol), [3 H]thymidine (56 Ci/mmol), Bolton-Hunter reagent (125 I; 2,000 Ci/mmol), and Omnifluor were purchased from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. N-acetyl-D-glucosamine, Triton X-100 (scintillation grade), DOC, and polyethylene glycol 6000 were from BDH Chemicals Ltd., Poole, England; Tween 20 and sodium cyanoborohydride were from Sigma Chemical

Co., St. Louis, Mo. Wheat germ lectin-Sepharose 6MB, AH-Sepharose 4B, dextran T-500, and PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Kodak XG-14 X-ray films were used in the autoradiography and fluorography analyses and were developed in Kodak D-19, both products from Eastman Kodak Co., Rochester, N.Y. Glutaraldehyde (25% for electron microscopy) was from E. Merck AG, Darmstadt, West Germany.

RESULTS

Comparison of procedures for plasma membrane isolation. Two methods were studied for plasma membrane isolation as described in Materials and Methods. The recoveries of protein and distributions of marker enzymes are shown in Table 1. Based on the greater recovery of protein, the higher enrichment of 5'-nucleotidase activity, the facts that the two-phase separation procedure is faster, less laborious, and more suitable in a preparative scale, this technique was chosen for further plasma membrane preparations (6). The purity of the plasma membranes prepared by this method was controlled in a phase-contrast microscope and electron microscope, and no contamination of intact cells, nuclei, mitochondria, or granular elements was observed (data not shown).

Solubilization of plasma membrane proteins. The efficiencies of membrane protein release by various functionally distinct solubilization techniques were compared (Table 2). Some extractions were performed in such a way that one solubilization technique was followed by another in order to release material from the nonextractable remainder of the previous pro-

TABLE 1. Comparison of methods for plasma membrane isolation^a

Method and sample	Protein recovery (%)	5'-Nucleotidase		Na ⁺ ,K ⁺ -ATPase		NADPH cytochrome c reductase		Recovery (%)
		Sp act (μ mol of PO ₄ ⁻³ /h and mg)	Relative sp act	Sp act (μ mol of PO ₄ ⁻³ /h and mg)	Relative sp act	Sp act (μ mol/h and mg)	Relative sp act	
Bosmann et al. (5)				ND		ND		
Homogenate	100	1.7 $\pm$ 1.1 (3)						
Supernatant	50.4 $\pm$ 36.2 (3)	1.2 $\pm$ 0.9 (3)	0.9 $\pm$ 0.4 (3)					
Fraction II ^b	1.5 $\pm$ 0.17 (3)	4.7 $\pm$ 1.8 (3)	3.6 $\pm$ 1.9 (3)					
Brunette and Till (7)								
Homogenate	100	1.4 $\pm$ 0.8 (6)		0.29 $\pm$ (5)		0.49 $\pm$ 0.09 (4)		
Supernatant	62.5 $\pm$ 6.0 (13)	1.4 $\pm$ 0.4 (3)	0.8 $\pm$ 0.2 (3)	ND		0.56 $\pm$ 0.18 (4)	1.17 $\pm$ 0.37 (4)	106 $\pm$ 23.6
Interphase ^c	3.9 $\pm$ 0.9 (23)	9.3 $\pm$ 4.9 (6)	7.3 $\pm$ 2.8 (6)	2.10 $\pm$ 1.1 (5)	10.5 $\pm$ 8.4 (5)	0.85 $\pm$ 0.27 (4)	1.72 $\pm$ 0.28 (4)	3.7 $\pm$ 1.8

^a Values are expressed as mean $\pm$ standard deviation; number of experiments is given in parentheses. NADPH, Reduced nicotinamide adenine dinucleotide phosphate; ND, not determined.

^b Described in Materials and Methods.

^c In the two-phase separation system, the plasma membranes are enriched in the interphase.

TABLE 2. *Receptor activity (RI) of solubilized plasma membrane proteins recovered by different procedures^a*

Extraction procedure	Protein recovery (%)	Amt of protein needed for 50% RI (μ g)	Amt of protein needed for 50% RI normalized to the 0.5% Triton X-100 extraction system (μ g)
3 M KCl	17.6 $\pm$ 3.0 (4)	150 (1)	3.1
3 M KCl-10% pyridine ^b	4.5 $\pm$ 0.8 (2)	ND	
3 M KCl-0.5% Triton X-100 ^b	46.0 (1)	84 (1)	1.8
10% <i>n</i> -Butanol	12.0 (1)	ND	
10% <i>n</i> -Pentanol	17.7 (1)	ND	
10% Pyridine	20.8 $\pm$ 4.7 (5)	74 $\pm$ 17.6 (2)	1.5 $\pm$ 0.4
10% Pyridine-10% <i>n</i> -pentanol ^b	0 (1)	ND	
1.0% DOC	69.7 (1)	59 (1)	1.2
0.1 % Triton X-100	25.2 (1)	NE (1)	
0.5 % Triton X-100	37.6 $\pm$ 4.9 (14)	48 $\pm$ 13.2 (25)	1.0 $\pm$ 0.2
1.0% Triton X-100	43.9 $\pm$ 6.9 (3)	52 $\pm$ 7.1 (2)	1.1 $\pm$ 0.2
0.5% Triton X-100 - PD-10 ^c		71 $\pm$ 26.9 (2)	1.5 $\pm$ 0.6
0.5 % Tween 20	18.8 $\pm$ 6.3 (3)	NE (10)	
1.0% Tween 20	30.6 $\pm$ 6.8 (4)	NE (5)	
Total membranes		86 $\pm$ 19.6 (11)	1.8 $\pm$ 0.4

^a The relative inhibition of virus attachment was calculated as described in Materials and Methods. Where applicable, values are followed by standard deviation, with the number or experiments given in parentheses. ND, Not determined; NE, no effect (highest amount of protein analyzed was 80 μ g).

^b A second extraction of the nonextractable remainder of the first was done as described in Materials and Methods.

^c Before analysis in the attachment assay, the protein extracts were deprived of free detergent by passage through a PD-10 column followed by extensive dialysis.

cedure. It is evident that the detergent methods released the highest quantities of membrane protein. The high-salt and the organic solvent procedures were one-third to one-half as efficient.

When we compared the total polypeptide contents of the high-salt and the detergent extracts in SDS-polyacrylamide slab gels, we found that the solubilization by detergents enriched a number of proteins compared with the starting material (Fig. 1). Qualitatively, all extracts displayed similar polypeptide patterns. The electropherogram also supported the general concept about the versatility of proteins constituting the protein moiety of the HeLa cell plasma membrane (8).

Identification of receptor-active material. Solubilized plasma membrane proteins obtained from the high-salt, organic solvent, and detergent extraction procedures were analyzed

for the presence of material interfering with the process of adenovirus attachment to HeLa cells. A prerequisite for this analysis was the development of an assay for receptor-active material and its subsequent normalization (see Materials and Methods). Initially, the kinetics of adenovirus attachment to HeLa cells was studied (Fig. 2). After approximately 40 min of virus-cell interaction, maximum attachment was attained. The increased values for the standard deviation of later measuring points could have resulted from physical stress on the cells after this time of incubation. The tendency of the function to decrease at later time points could have been due to the fact that virions at this stage of incubation had entered the interior of the cells (26) and thus avoided detection, since the samples were assayed for radioactivity directly and not hydrolyzed before analysis. The basis for the normalized relative inhibition calculations was the dose-response curve (Fig. 3). Compared with total membrane content, a significant amount of receptor-active material (i.e., material interfering with virus attachment) was present in the 0.5% Triton X-100 extracts. Resuspension of the nonextractable remainder provided a sample with low ability to inhibit virus attachment, and although extreme quantities were used, only a maximum relative inhibition of around 25% was achieved. Table 2 shows the efficiencies of various other plasma membrane protein extracts in the inhibition assay. The Triton X-100 procedure using 0.5% detergent gave a 50% relative inhibition of virus attachment, using the lowest amount of extracted protein. Extraction with this concentration of detergent also made it possible to perform complete dose-response analyses without removing the detergent (see Materials and Methods). To simplify the comparison of Triton X-100 extracts with other extracts, all estimates were normalized to the Triton X-100 (0.5%) system (Table 2). Although the DOC procedure gave good production of receptor-active material, it was not considered for further studies because it could strongly interact with proteins irreversibly. The 3 M KCl extraction method produced an extract with poor inhibitory effect. We tried to optimize the Triton X-100 extraction procedure by first removing membrane components of low interfering ability with 3 M KCl and thereafter apply the Triton X-100 system to the nonsolubilized remainder. This sequence of extractions yielded an extract with only half the efficiency of the standard Triton X-100 (0.5%) procedure (Table 2).

Identification of polypeptides attaching to adenovirions. Since the Triton X-100 (0.5%) method produced an extract with the highest inhibitory effect on virus attachment to HeLa

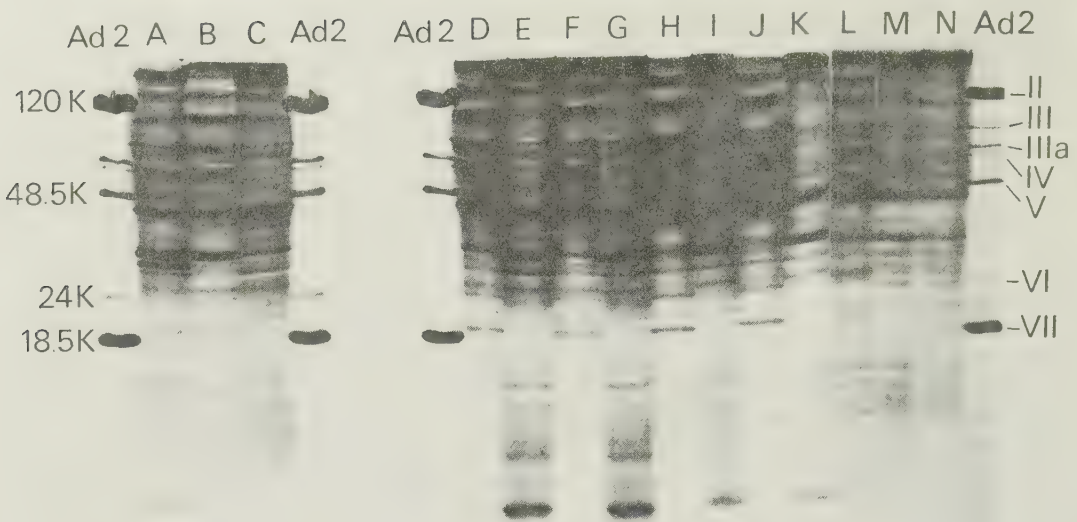


FIG. 1. Qualitative comparison in a stained SDS-polyacrylamide slab gel electropherogram of solubilized HeLa cell plasma membrane proteins. Each sample well was loaded with approximately the same amount of protein (75 μ g), and the gels were electrophoresed for a total time of 150 min, starting at 80 V for the first 20-min period and then increasing to 100 V. The contents of the lanes are as follows: (Ad2) marker Ad2 virions with indicated positions and molecular weights of major structural polypeptides as described by Anderson *et al.* (1); (A) 3 M KCl extract; (B) 0.5% Triton X-100 extract of the nonextractable remainder after a 3 M KCl extraction; (C) nonextractable remainder after consecutive extractions with 3 M KCl and 0.5% Triton X-100; (D) 0.5% Triton X-100 extract; (E) nonextractable remainder after a 0.5% Triton X-100 extraction; (F) 1% Triton X-100 extract; (G) nonextractable remainder after a 1% Triton X-100 extraction; (H) 0.5% Tween 20 extract; (I) nonextractable remainder after a 0.5% Tween 20 extraction; (J) 1% Tween 20 extract; (K) nonextractable remainder after a 1% Tween 20 extraction; (L) 1% DOC extract; (M) nonextractable remainder after a 1% DOC extraction; (N) total plasma membranes used for the various extractions. All extractions were performed as described in Materials and Methods except that the sample of lane M was recovered after centrifugation at $3,000 \times g$ for 30 min.

cells, this technique was chosen for further investigations. Solubilized and *in vitro* 125 I-labeled plasma membrane proteins were incubated with highly purified Ad2 virions as described in Materials and Methods. The incubated mixtures were subsequently layered onto sucrose gradients and sedimented as described. Reproducibly about 0.3% of the added radioactivity cosedimented with the virus particles in the presence of 0.037% Triton X-100 (Fig. 4). If the detergent concentration of the incubation mixture and gradient was increased to 0.5% Triton X-100, about 0.2% of the total radioactivity still remained associated with the virus. An additional increase of the detergent concentration up to 1% did not further reduce the amount of material attached to virions. In some instances, a low degree of virion and protein aggregation was discerned (0.06% of the total radioactivity) in the presence of both 0.037 and 0.5% Triton X-100. However, the majority of sedimenting labeled protein was always located in the region of the virus control. When 1 or 3% BSA was added to the incubation mixtures and gradients, we observed no mem-

brane proteins specifically cosedimenting with virions. When this large amount of protein was added (50 to 150 times more BSA per virus particle than membrane protein per virus particle), virus and membrane protein seemed to aggregate nonspecifically. Approximately 2.4% of the extracted membrane proteins were aggregated throughout the entire gradient when 3% BSA was used, compared with about 0.7% with 1% BSA.

Fractions from the sucrose gradient analyses of protein cosedimenting with virions were pooled as indicated in Fig. 4 and subjected to SDS-PAGE analysis as described in Materials and Methods. About 13 polypeptide bands were found associated with virions in the presence of 0.037% Triton X-100 (Fig. 5A). One of the predominant polypeptides had a molecular weight of approximately 40,000. The addition of 0.2 M NaCl had no effect on the qualitative pattern of polypeptides associated with the virus particles (data not shown), although the amount of proteins associated with virions was reduced to about 0.1%. If the detergent concentration was

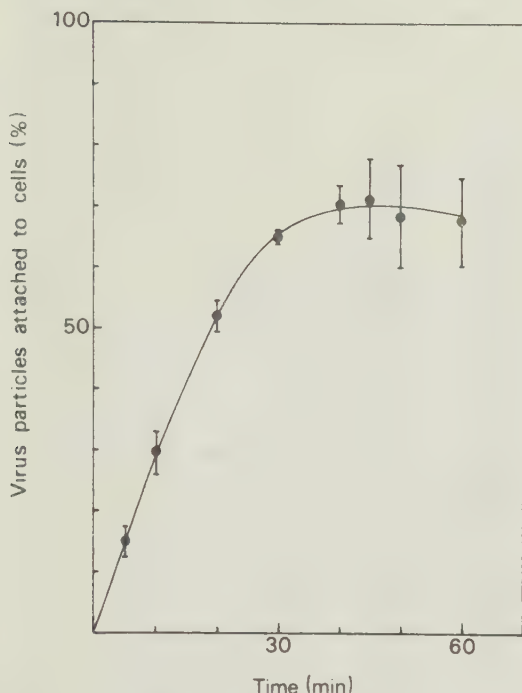


FIG. 2. Kinetics of adenovirus attachment to HeLa cells. Preincubated virus particles were mixed with HeLa cells, and samples were withdrawn at intervals after incubation began. The amount of radioisotope-labeled virus particles attached to cells was measured as described for the control series in Materials and Methods. Six separate experiments were performed, and the standard deviation is indicated for each measuring point.

increased to 0.5%, the 40,000-dalton polypeptide was the predominant species associated with virions, together with four faintly observable polypeptides (Fig. 5B). This 40,000-dalton polypeptide was probably identical to the protein species adsorbed with the highest affinity to the Ad2-AH-Sepharose 4B matrix (see below).

Lectin chromatography of solubilized plasma membrane proteins. Triton X-100 (0.5%)-solubilized and [3 H]formaldehyde-labeled membrane proteins were fractionated on wheat germ lectin-Sepharose 6MB as described in Materials and Methods. Radioactivity measurements reproducibly established that 5% of the total amount of protein material applied was retained by the column (pool B, Fig. 6).

Immobilized Ad2 as an adsorbent. Because adenovirus particles are fragile (25, 35), they must be stabilized before being immobilized and used as an adsorbent. Proteins of the virus particles were therefore cross-linked with glutaraldehyde and immobilized as described in Materials and Methods. These procedures pre-

served the ability of virions to interact with antifiber immunoglobulin G. Virions immobilized with no spacer reacted to a limited extent with antifiber antibodies, whereas the introduction of a six-carbon spacer produced a functional adsorbent (not shown).

Unretained and retained fractions of the Triton X-100-solubilized and radioisotope-labeled plasma membrane proteins from the wheat germ lectin-Sepharose 6MB chromatography step were analyzed in a column of immobilized Ad2 virions. Figure 7 shows the elution pattern when unretained material from the lectin chromatography (pool A, Fig. 6) was applied to the column. Of the radioactive material, 95% had no affinity for Ad2 and could be washed out with WGA buffer. Of the remaining 5% of radioactivity, 3% was eluted with WGA buffer containing 0.5 M NaCl, whereas the radioactivity still bound was eluted only after a drastic decrease in pH (see Materials and Methods).

When membrane proteins possessing *N*-acetyl-D-glucosaminyl residues (pool B, Fig. 6) were fractionated on the Ad2 column, a totally different pattern was obtained. Only 41% of the total radioactivity could be washed out unadsorbed with WGA buffer (Fig. 7B). Of the remaining

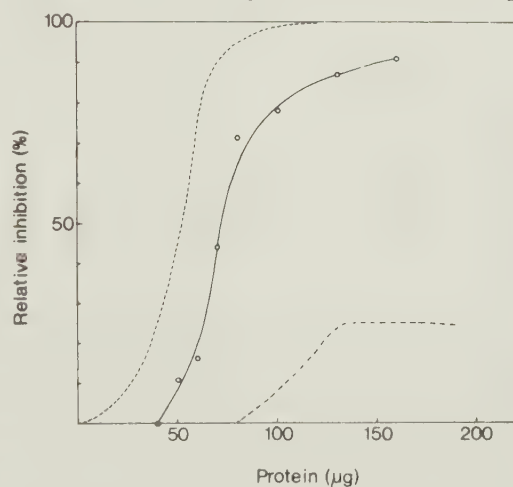


FIG. 3. Relative inhibition of virus attachment to HeLa cells in the presence of various amounts of presumptive receptor-active material. Relative inhibition in the presence of (---) plasma membrane proteins extracted with 0.5% Triton X-100 (the mean function was constructed based on three separate experiments with six measuring points per experiment); (—○—) total plasma membranes of HeLa cells (one experiment was performed); and (-.-.-) dissolved protein content from the nonextractable remainder after a 0.5% Triton X-100 extraction. The mean function was constructed based on two separate experiments with six measuring points per experiment.

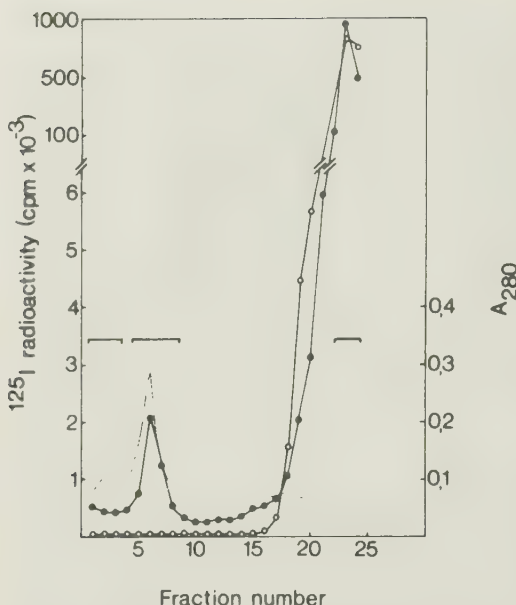


FIG. 4. Sucrose gradient analysis of HeLa cell plasma membrane proteins adsorbed to adenovirus particles. Symbols: (—●—) Radioactivity of Triton X-100 (0.5%) solubilized plasma membrane proteins, ^{125}I -labeled *in vitro* and mixed with Ad2 virions; (—○—) radioactivity of solubilized, iodine-labeled proteins analyzed for self-aggregation in a separate centrifuge tube; (...) UV absorbance (260 nm) of Ad2 particles sedimented in a separate centrifuge tube as a sedimentation control for the system. A typical experiment with 0.037% Triton X-100 in incubation mixtures and gradients is shown, details of which are described in Materials and Methods. Sedimentation was from right to left. The horizontal bars indicate fractions pooled for further analysis (see Fig. 5).

59%, 38% was eluted at high ionic strength (0.5 M NaCl) and 21% was eluted at low pH (2.6). This means that about one-fifth of the wheat germ lectin-adsorbed proteins had high affinity for Ad2, which further corresponds to about 1% of the totally solubilized membrane protein content. This figure should be compared with the 0.3% of solubilized membrane proteins adsorbed to Ad2 virions in the sucrose gradient system.

Polypeptide analysis of the virus-retaining material. From the virus affinity chromatograms, the different peak fractions were pooled as indicated in the Fig. 7 and further analyzed on SDS-polyacrylamide slab gels (Fig. 8). Because of the low amount of radioactivity applied, patterns for sample wells with several polypeptides were difficult to observe. The pooled fractions from pool B in the lectin chromatography step (Fig. 6) and with high affinity for Ad2 virions (pool B3 of Fig. 7B) revealed one heavily

and one poorly labeled polypeptide band with apparent molecular weights of 42,000 and 40,000, respectively. The analogous fractions derived from pool A (Fig. 6 and 7A) showed two equally labeled polypeptide bands with the same apparent molecular weights as those of pool B3 (Fig. 7B).

DISCUSSION

The plasma membrane fraction of HeLa cells has been isolated preparatively, and by means of various extraction procedures some information has been obtained on the nature of the Ad2 attachment proteins and their association with the plasma membrane.

Assuming that the components that are important for the initial attachment step of viruses to cells are loosely associated surface entities of the plasma membrane, high-salt conditions should be effective in releasing this material. The 3 M KCl extraction procedure used here is well established for these purposes (33), but a high-salt extract of plasma membranes had little effect in the relative inhibition assay (Table 2). Presumably due to denaturing conditions of the high-salt procedure or the removal of a cooperating attachment factor, a consecutive Triton X-100 detergent extract also had little effect in the receptor assay. The results indicate that the attachment proteins are localized as integral entities of the plasma membrane. This is further supported by the fact that removal of the free detergent from a 0.5% Triton X-100 extract decreased the inhibitory activity (Table 2).

That the assayed components were integrated structures of the membranes was further indicated by the observation that an extract of the nonionic detergent Tween 20 did not display any measurable receptor activity (Table 2). One explanation for the lack of receptor release with this detergent could be the higher hydrophilic-lipophilic balance value for Tween 20 (16.7) than for Triton X-100 (13.5). It is generally believed that hydrophilic-lipophilic balance values above 14.5 are associated with lower ability to solubilize integral membrane proteins (15, 17). Of course, the different configurations of the detergents, i.e., size and structure of the polar and apolar moieties of the detergent molecules, could also contribute to the distinct extraction properties. Conformational changes of the attachment proteins cannot be ruled out, but to circumvent this possibility the receptor assay was developed and an extraction procedure was eventually selected that yielded receptor-active material in a soluble state.

Hennache and Boulanger (18) described the isolation of proteins interfering with the attach-

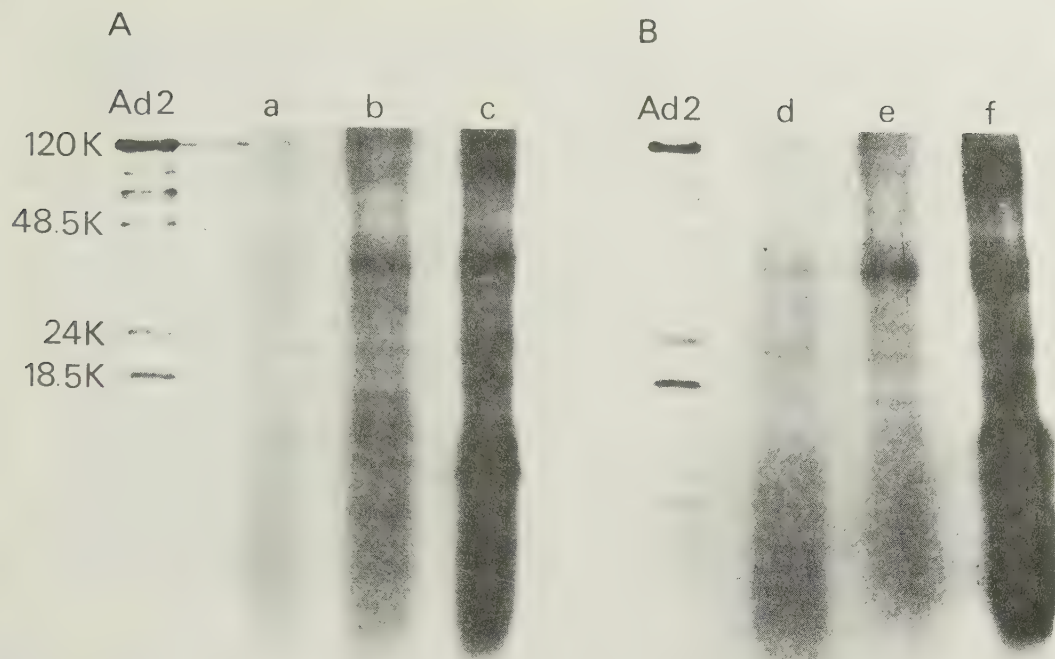


FIG. 5. Autoradiogram of an SDS-polyacrylamide slab gel electropherogram of ^{125}I -radiolabeled membrane proteins recovered from a sucrose gradient sedimentation analysis of proteins adsorbed to Ad2 particles. (A) Analysis of membrane proteins recovered from a sucrose gradient sedimentation in the presence of 0.037% Triton X-100 (Fig. 4). (a) Fractions 1 through 3; (b) fractions 5 through 9; (c) fractions 23 through 24. (B) Sucrose gradient analysis in the presence of 0.5% Triton X-100. Fractions from the sedimentation chromatogram were pooled as in (A), giving sample slots d, e, and f. ^{14}C -amino acid-labeled Ad2 virus was used as a polypeptide marker with indicated positions and molecular weights of major structural proteins as described by Anderson et al. (1). The gels were run as described in the legend of Fig. 1.

ment of Ad2 to KB cells. Fibers and total penton structures of the virions, immobilized by various techniques, were used as adsorbents. By using total Ad2 virions, we have allowed for the subsequent identification of all possible membrane components of stronger as well as weaker affinity for all surface structures of the intact Ad2 virion.

We noticed a quantitative difference in the amount of extracted proteins associated with virions under various conditions. In the sucrose gradient system, the attachment efficiency ranged between 0.2 and 0.3% when Triton X-100 was present at 0.5 and 0.037%, respectively. Membrane proteins were in these instances added in amounts ranging from 2×10^{-10} to 3×10^{-10} μg per virion. Ten- to 100-times-lower ratios of protein over virions were used in the immobilized system, and under these conditions as much as 3% of the solubilized protein content was adsorbed. Of this material, one-third (i.e., 1%) was retained with high affinity. The calculations were based on pertinent fractions, taking

into account all possible technical considerations. Meager et al. (31) and Hughes and Mautner (21) have suggested the involvement of glycoproteins during virus-cell interaction, and our preliminary data also suggest that glycosylated components are of interest. Therefore, fractionation on WGA-Sepharose was done to drastically concentrate the glycoprotein content of the total membrane extracts; in this way, samples relatively rich in receptor activity were obtained for the subsequent fractionations on the immobilized virion particle matrix. It should be emphasized that this prefractionation was not done before the assays in the sucrose gradient system, and this fact could explain the lower yield of virus-attaching proteins in the gradient analyses compared with the Ad2 matrix system. Supporting data for the idea of nonspecific protein competition may also come from experiments where large quantities of added proteins irrelevant for the virus-cell attachment step, e.g., BSA, strongly affected the attachment of membrane

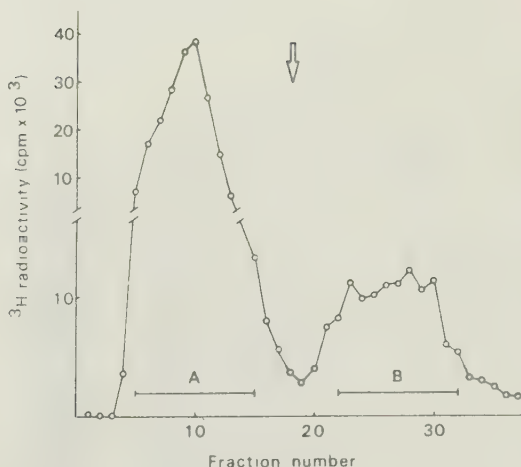


FIG. 6. Wheat germ lectin-Sepharose 6MB chromatography of Triton X-100-solubilized and *in vitro* [^3H]formaldehyde-labeled plasma membrane proteins of HeLa cells. A typical experiment is shown in which 1.4×10^7 cpm of radioactivity was applied to the column and eluted as described in Materials and Methods. The arrow indicates the start of elution with WGA buffer containing *N*-acetyl-D-glucosamine (100 mg/ml). The horizontal bars (A and B) represent fractions pooled for further studies (see Fig. 7).

proteins to virions. These findings are contradictory to the results of Hughes and Mautner (21), who in their not fully comparable system of purified fibers and membrane components were not able to affect the fiber-receptor recognition.

The analyses in sucrose gradients and on immobilized virus particles apparently show at least two types of affinity of membrane proteins to virus particles. We focused here on the high-affinity fraction, but it is possible that proteins with lower affinity for viruses still may be involved in the attachment step. The high-affinity fraction obtained by the two procedures is dominated by a radiolabeled polypeptide with an apparent molecular weight of 42,000. One of the Ad2 attachment polypeptides on KB cells enriched by Hennache and Boulanger (18) displayed the same molecular weight (18).

Based on the elution profiles from the affinity chromatographies on WGA and Ad2 matrices (Fig. 6 and 7), we suggest that the high-affinity proteins contain *N*-acetyl-D-glucosaminyl residues. This means that the indicated molecular weights may be uncertain, because SDS-PAGE is not the optimal system to determine the molecular weight of glycoproteins (14). The autoradiogram patterns for pools A3 and B3 (Fig. 8) were very similar, and the visible bands probably corresponded to the same polypeptide. However, with respect to the different affinities for WGA, the carbohydrate moieties of the polypeptides in

A3 and B3 must have been distinct, since the elution patterns were not caused by overloading artefacts during chromatography. The different behaviors may be due to a partial degradation of the carbohydrate moiety or incomplete glycosylation during the biosynthesis of the polypeptides recovered in pool A3 (28).

Since it has been reported that the receptors for Ad2 are sparsely represented on human cell plasma membranes (21), we have worked with membrane proteins radiolabeled *in vitro* to identify the components of the attachment site. It is not possible to obtain the same specific radiolabeling of all polypeptides in the plasma mem-

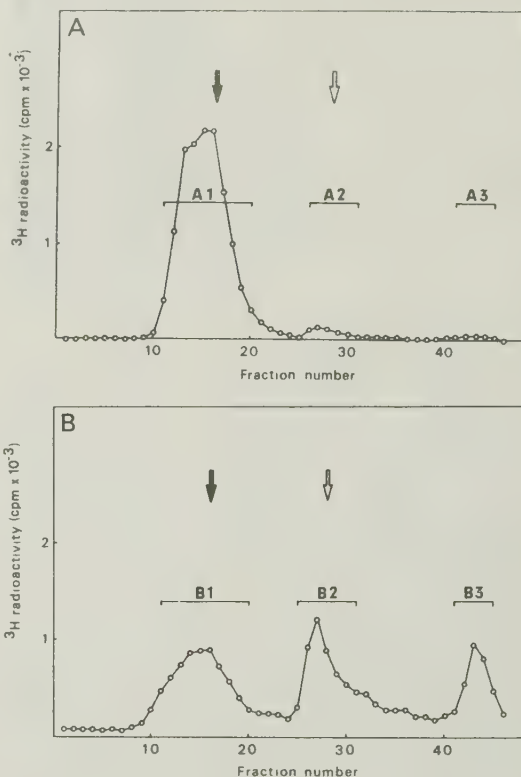


FIG. 7. Affinity chromatography on an immobilized Ad2 matrix. (A) Pool A from the wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was applied to a column of cross-linked Ad2 virions immobilized to AH-Sepharose 4B and fractionated as described in Materials and Methods. The filled arrow indicates the start of elution with WGA buffer containing 0.5 M NaCl, and the open arrow indicates the start of elution with 0.1 M NaCl-0.037% Triton X-100 in 0.1 M glycine-hydrochloride buffer, pH 2.6. The horizontal bars represent the peak fractions pooled and are denoted A1, A2, and A3 according to elution order. (B) Pool B from the previous wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was fractionated as described above.

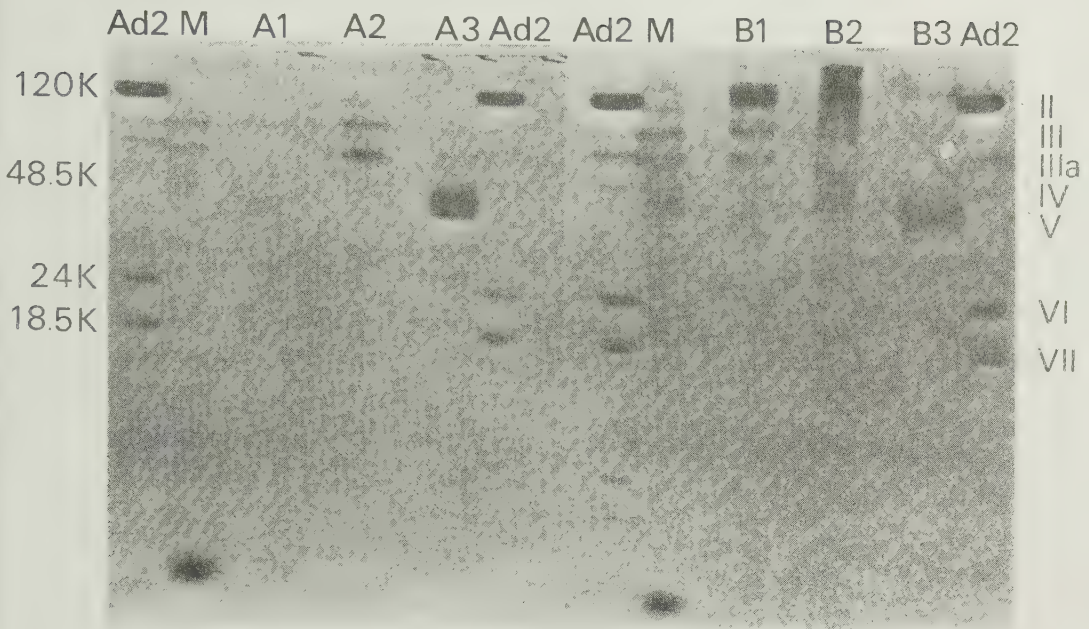


FIG. 8. SDS-PAGE of pooled fractions from an immobilized Ad2 affinity chromatography separation of prefractionated HeLa cell plasma membrane proteins labeled *in vitro*. Samples recovered as described in the legend of Fig. 7 were applied to each sample well in equal amounts of radioactivity (6,000 cpm). Lane M is total, solubilized, and *in vitro* [^3H]formaldehyde labeled plasma membrane proteins. A1, A2, and A3 are the pooled fractions of the separation demonstrated in Fig. 7A; B1, B2, and B3 are the corresponding fractions of the separation of Fig. 7B. A ^{14}C -amino acid-labeled preparation of Ad2 was used as a polypeptide marker with indicated positions and molecular weights of major structural polypeptides as described by Anderson *et al.* (1). The gels were run as described in the legend of Fig. 1 and subsequently analyzed by fluorography.

brane. One must therefore consider that some polypeptides may escape being labeled. Thus, our preliminary results with unlabeled HeLa cell plasma membrane proteins reveal similar patterns when chromatographed on WGA and Ad2 matrices. However, in these instances the A3 and B3 pools, upon SDS-PAGE analysis, display distinct polypeptide patterns after staining of the electropherograms. Moreover, it has been demonstrated that very low quantities of unlabeled proteins of pool B3 interfere with the Ad2 attachment to cells (R. Persson, U. Svensson, and E. Everitt, manuscript in preparation).

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Virus Receptor Interaction in the Adenovirus System II. Capping and Cooperative Binding of Virions on HeLa Cells

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Adenovirus type 2 attachment to HeLa cells was analyzed under controlled conditions. The temperature-dependent attachment kinetics revealed an inflection point at around 20°C, and above this temperature the increase of the rate was doubled. In multiplicity dependence experiments, the attachment exhibited positive cooperative binding at 37°C. This binding pattern was inhibited by low temperatures and prefixation of cells with 0.015% glutaraldehyde. Attachment of rhodamine-labeled virions revealed capping of the particles on 15% of the cells at 37°C. Capping was inhibited by low temperatures, glutaraldehyde fixation of cells, and treatment with cytochalasin B, azide, and 2-deoxyglucose. Consequently, we propose that the adenovirus type 2 attachment to cells leads to rearrangements in the plasma membrane, resulting in cooperative binding and capping of the virus particles.

The important first step for a successful replication of animal virus includes attachment, penetration or internalization, and uncoating. Attachment to cells is believed to involve an interaction between viruses and specific components of the plasma membrane. Lonberg-Holm and Philipson (18) have outstandingly surveyed the early interactions between animal viruses and cells, and review articles which focus on recent achievements within the field continually appear (3, 5, 19).

For clarity, we have adopted the nomenclature defined by Lonberg-Holm (17): virus attachment proteins are the structural components of the virion recognizing a specific cellular receptor, cellular receptor units (CRU) are cellular molecules recognizing one virus attachment protein, and cellular receptor sites (CRS) are cellular structures composed of one or more CRU involved in the binding of a virus particle.

In the adenovirus system, the well-characterized fiber protein has been identified as the virus attachment protein in the sense that isolated fibers adsorbed to cells efficiently block a subsequent virion attachment (26). Moreover, antibodies against purified fiber antigen quantitatively precipitate virions (27) and consequently inhibit infection as assayed by the fluorescent focus technique, but curiously not by the plaque assay (23, 24). The maximum number of virions which can attach to a cell is limited by the number of available CRS. Since in the adenovirus system the CRU/CRS ratio is in the range of 10 to 100 (26), either the isolated fiber molecules

may recognize attachment units unavailable for the binding of larger virions, or virion attachment implies the need for engagement of several CRU. This multivalency of CRS may be achieved during the mere process of virion attachment, which might cause physical alterations of the lipid bilayer, as well as provoke changes in the lipid composition within the microenvironment of the CRS (17).

Kohn (15) has suggested that attachment of viruses or ligands to possibly cytoskeletally anchored CRU cross-links the receptors and thus modifies the interaction of the latter with the lipid phase of the membrane, and this is expressed as a change in fluidity. Interestingly, it has been shown for some nonenveloped viral systems that a decrease in microviscosity is induced upon virus attachment, an attachment-specific phenomenon since it only appeared in cells with intact receptors and in cells permissive with regard to the attachment step (16). As a consequence of virion adsorption to KB cells at low temperatures, Hennache et al. (10, 11) demonstrated that adenoviruses induce a rearrangement and clustering of intramembranous particles and a change in the distribution of sterols.

In light of the preceding information, we have in this study made attempts to unravel the mechanisms behind the adenovirus type 2 (Ad2) attachment to HeLa cells by manipulating the permissive system through changing a physical parameter such as the temperature, chemically stabilizing the cell surface by fixation, and adding physiologically interacting drugs and reagents.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at cell densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenoviruses were propagated, radioisotope labeled, and purified as described previously (7).

Attachment studies. The general procedure of virion attachment was as described earlier (35). HeLa cells were washed in phosphate-buffered saline (PBS) once or three times for experiments in unfixed or glutaraldehyde (GA)-fixed series, respectively. [3 H]thymidine-labeled virions (3.0×10^6 cpm per 10^{12} virions) were allowed to attach to cells in PBS at a density of 5×10^7 cells per ml.

In temperature dependence experiments, cells and viruses were incubated in PBS at temperatures between 2.8 and 37°C. In all experiments, cells were equilibrated at the relevant temperature for 5 min before viruses were added. The number of virions per cell was kept at a constant multiplicity of infection (MOI) of 340. Samples were withdrawn at 5, 10, 20, 30, 45, and 60 min after the addition of virus, diluted 10 times in PBS, and gently sedimented for 25 s. The radioactivity confined to cell pellets and supernatants was measured and used for calculating the degree of virion attachment.

In multiplicity dependence experiments, ratios of viruses over cell numbers were between 50 and 20,000. Incubation periods for cell and virus mixtures were 45 min for unfixed and GA-fixed cells at 37°C and 3 h for unfixed cells at 2.8°C. After incubation, samples were diluted six times in PBS, sedimented, and assayed for radioactivity as described above.

The possible effect of cytochalasin B (15 to 60 μ g/ml) colchicine (0.1 to 0.4 mM), sodium azide (10 to 50 mM), and 2-deoxyglucose (10 to 50 mM) on the virion attachment was studied. Before virus addition, cells were preincubated for 10 min at 37°C with the appropriate reagent. Ad2 was added at an MOI of 340, and incubations were continued for 60 min. Samples were withdrawn at intervals, and virus attachment was assessed as above. The same reagents were used in the fluorescence studies.

GA fixation of cells. Various concentrations of GA were tested to obtain an optimal stabilization of cells with a concomitant minimal influence on the properties of the CRS. Cells, washed three times in PBS, were suspended in PBS at a concentration of 10^6 per ml. Freshly prepared 2.5% GA in PBS was added to yield final concentrations of 0.001 to 0.6%. Cell suspensions were incubated at 4°C for 30 min under slow agitation, after which the cells were sedimented and resuspended, at the density above, in PBS containing 0.05 M neutralized ethanolamine. After a 30-min incubation, cells were washed three times in PBS and finally suspended at a density of 5×10^7 per ml. The status of the cells was studied accordingly.

Virion attachment at 37°C revealed that cells fixed with 0.01% GA were almost unaffected, with a reduction in attachment of less than 10%, whereas at 0.06% GA the reduction amounted to 90%. Swelling of cells in hypotonic buffer (10 mM Tris-hydrochloride buffer containing 10 mM KCl, 2 mM $MgCl_2$, and 2 mM β -mercaptoethanol, pH 7.3) for 15 min at 22°C, detergent

treatment (0.1% Triton X-100 in PBS) of cells for 60 min at 22°C, and ultrasonic treatment of cells for 5 s on ice demonstrated that cells stabilized with 0.01% GA appeared to be unaffected by the various treatments, as judged from microscopic examination. A concentration of 0.015% GA was chosen in our subsequent fixation studies of cells, and at this concentration virus adsorption was reduced at the most by 30%.

Rhodamine labeling of Ad2 virions. Tetraethylrhodamineisothiocyanate (TRITC) was covalently linked to Ad2 virions as follows. Immediately before conjugation, highly purified virions were filtered through a PD-10 column, equilibrated in 0.1 M $NaHCO_3$, pH 8.7. The concentration of recovered viruses was 3.6 optical density units per ml, corresponding to 1.01 mg of virus protein (22). TRITC in 0.1 M $NaHCO_3$ -ethanol (1:1) at a concentration of 1.0 mg/ml was added to the virus suspension at a ratio of TRITC to virus protein of 0.05. The mixture was incubated at 26.5°C for 3 h, after which the conjugated virions were separated from free TRITC in a PD-10 column equilibrated in PBS, with a recovery of 75%. Spectrophotometric measurements at 560 nm revealed a number of TRITC molecules per virion in the range of 5×10^3 to 7×10^3 . No disintegration of conjugated virions was discernible after sedimentation analyses in glycerol gradients. The capacity of TRITC-labeled Ad2 to attach to cells was reduced by 10 to 15%, and unconjugated Ad2 particles competed quantitatively with TRITC-labeled Ad2 for receptor sites on the cells.

Attachment studies with TRITC-conjugated Ad2. Cells were carefully washed and suspended in PBS at a density of 5×10^7 per ml. TRITC-conjugated Ad2 was added at an MOI of 3,000 and adsorbed at 2.8°C for 60 min. After attachment, cells were washed with PBS and further processed in one of the following ways.

(i) Cells were fixed with GA directly after the initial attachment step. (ii) cells were incubated further at 37°C for 0.5, 1, 2, 5, 10, 20, 40, and 60 min and subsequently fixed. (iii) Cells were fixed and further incubated at 37°C for 60 min. TRITC-conjugated Ad2 was also added to prefixed cells and incubated at 2.8°C for 60 min.

In addition, attachment of TRITC-conjugated Ad2 was studied in the presence of sodium azide (10 and 30 mM), cytochalasin B (15 μ g/ml), colchicine (0.1 mM), and 2-deoxyglucose (10 mM). The virions were added to cells together with one of the reagents and incubated for 60 min at 2.8°C. After removal of unadsorbed virus with PBS, cells were suspended in PBS containing the indicated concentrations of reagents and further incubated at 37°C for 10 min. Immediately after the second incubation, the cells were fixed with 0.015% GA. Analyses were made in a Zeiss fluorescence microscope by using epifluorescence with BP 546, FT 580, and LP 590 filters. For photography, Ilford HP 5 films were used.

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were done in a cocktail of toluene-methanol (1:1) with 0.4% Omnifluor. A Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) was used to determine radioactivity.

Chemicals and reagents. Eagle minimal essential medium, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. [3 H]thymidine (74 Ci/mmol) and Omnifluor

were purchased from New England Nuclear Chemicals GmbH, Dreieich, West Germany. Cytochalasin B, colchicine, sodium azide, and 2-deoxyglucose were from Sigma Chemical Co., St. Louis, Mo. Rhodamine B isothiocyanate and ethanolamine were obtained from BDH Chemicals Ltd., Poole, England, and GA (25% for electron microscopy) was from E. Merck AG, Darmstadt, West Germany. PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

RESULTS

Temperature dependence of Ad2 attachment.

The attachment of Ad2 to cells was analyzed at several temperatures ranging between 2.8 and 37°C. To minimize fluctuation effects due to the cellular status, all samples were analyzed within 1 day and with material obtained from the same cell harvest. The virus attachment was highly dependent on the incubation temperature (Fig. 1). Also depending on the incubation temperature, maximum values of attachment were reached after different periods of incubation. These maximum values varied from around 80% attachment after 30 to 40 min at 37°C to around 45% after 3 h at 2.8°C (data not shown).

From the slopes of the curves in Fig. 1, the initial rates of viral attachment were calculated. Figure 2 shows that the attachment rates were strongly dependent on the incubation temperatures, with a shift at around 20°C, leading to a doubling of the rate increase above this tempera-

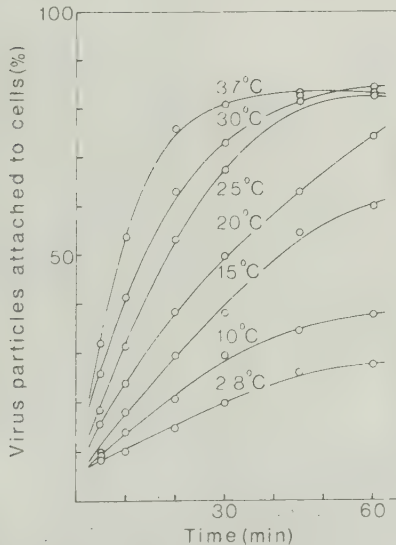


FIG. 1. Temperature dependence of the Ad2 attachment kinetics. Radioactive Ad2 virions were added to cells equilibrated at the appropriate temperature and further incubated. Samples from each incubation mixture were withdrawn at the indicated times, and the attachment of virions was determined as described in the text. The circles represent the mean values of attached viruses in two separate experiments.

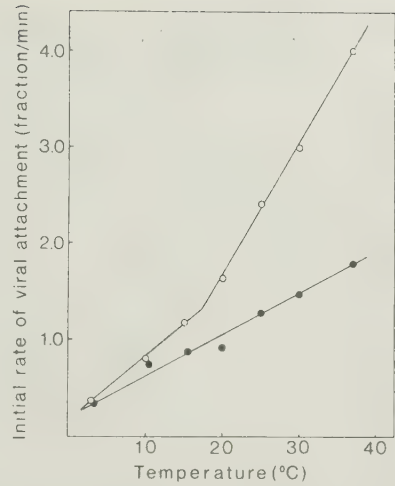


FIG. 2. Temperature dependence of the initial rate of adenovirus attachment. The initial rates of viral attachment at the different temperatures were calculated from the slopes in Fig. 1 to give the fraction of adsorbed virions per minute (○). ●, Attachment data from an analogous experiment with GA-fixed (0.015%) cells.

ture. For comparison, an analogous curve was made for cells fixed with 0.015% GA. The slope of this curve resembled the one for unfixed cells below 20°C, but revealed no shift in the increase in attachment rates above 20°C.

Temperature dependence of attachment of rhodamine-labeled Ad2. Ad2 particles conjugated with the isothiocyanate derivative of rhodamine were employed to visualize the early events of virion attachment. Due to the high specific fluoro-chrome labeling of virions, MOIs of 3,000 particles per cell were used to give a moderate number of attached virions (ca. 20%). Cells incubated with TRITC-conjugated Ad2 at 2.8°C for 60 min and subsequently fixed with 0.015% GA demonstrated a granular and patchy fluorescence pattern, uniformly distributed over the cell surface (Fig. 3A). This general pattern was not altered if fixed cells were transferred to 37°C and further incubated for 60 min.

The same pattern was also observed if cells were fixed before virion attachment and incubated as above. However, if cells with virions adsorbed at 2.8°C were transferred to 37°C, without prior fixation, the fluorescence became clustered and polarized to one end of the cells at a frequency of around 15% (Fig. 3B and C). Capping of labeled virus was evident as early as 30 s after the temperature shift. Approximately 20 min after the shift, the fluorescent clusters appeared less pronounced and became somewhat diffuse (Fig. 3D), possibly due to translocation of virions into the cytoplasmic region of the cells.

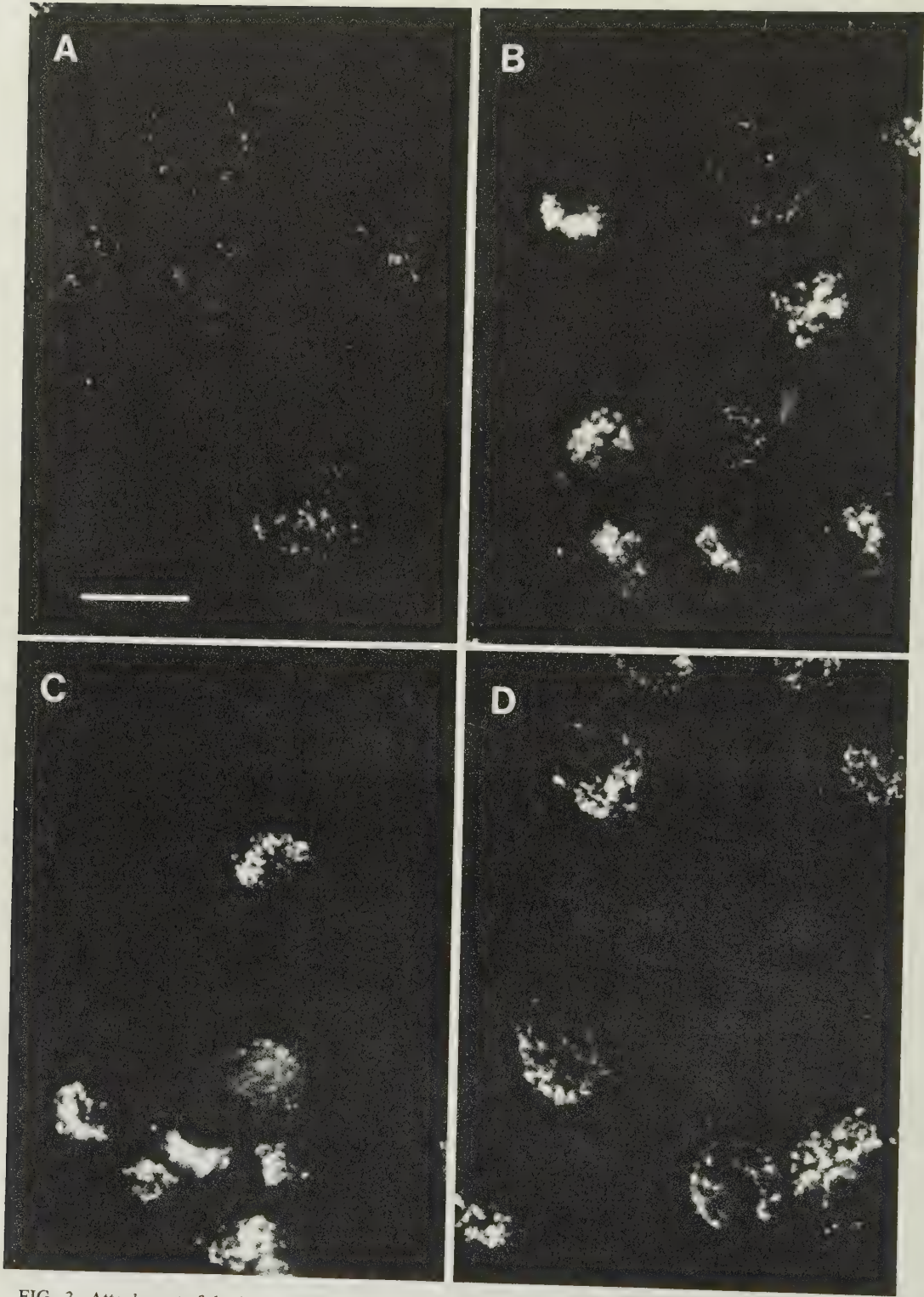


FIG. 3. Attachment of rhodamine-labeled Ad2 virions. Rhodamine-labeled virus was added to cells at an MOI of 3,000 at 2.8°C for 60 min. After removal of unadsorbed virus (80%), cells were transferred to 37°C and incubated for increasing periods of time. Samples were removed, subsequently fixed with GA (0.015%), and

Influence of drugs and metabolic inhibitors on virion attachment. Metabolic inhibitors (NaN_3 and 2-deoxyglucose) and drugs interfering with the cytoskeletal elements (cytochalasin B and colchicine) were analyzed with respect to possible effects on virion attachment. The reagents were added to cells 10 min before the addition of radiolabeled virus, and attachment was continually measured during a period of 60 min at 37°C. Azide, cytochalasin B, and colchicine were negative or reduced the final attachment by less than 10%, whereas 2-deoxyglucose at 50 mM reduced attachment by 20% as compared with control cells.

Although exhibiting only minor effects on viral attachment kinetics, the above reagents were also used to study the possible influence on the distribution of virions attached to the cell surface. In these instances, the reagents were added to cells at 2.8°C and incubated along with the TRITC-labeled virions for 60 min. After removal of unadsorbed virus with PBS, reagents were added again before the cells were transferred to 37°C for 10 min. After the second incubation, all cells were fixed with 0.015% GA, and 300 cells from each series were examined. The already-mentioned extent of capping to around 15% in unfixed cells at 37°C was inhibited after the addition of sodium azide (10 and 30 mM), cytochalasin B (15 $\mu\text{g}/\text{ml}$), and 2-deoxyglucose (10 mM) and thus equalled the background level of 2% for cold-treated or GA-fixed cells. Colchicine treatment of cells (0.1 mM) had no effect on capping.

MOI dependence of Ad2 attachment. Ad2 was added to HeLa cells at different MOIs ranging from 50 to 20,000 virions per cell. The virus-cell mixtures were incubated at 37 and 2.8°C for 45 min and 3 h, respectively. Virus attachment was studied on both GA-fixed and unfixed cells. Figure 4 shows the number of bound viruses at various MOI inputs. The curves reveal typical saturation patterns, demonstrating the virus-binding capacity of each cell. In this particular experiment, the GA fixation reduced the number of available CRS to 60% of the control cells. This was an exceptional outcome of the GA treatment, which normally did not display this CRS-reducing effect. However, these results are shown for the sake of comparison, since all temperature- and MOI-dependent experiments described in this communication were derived from the same cell harvest and performed within 1 day.

After conversion of the results into Scatchard plots, a positive cooperative binding at virion

attachment was reproducibly discernible for untreated cells at 37°C (Fig. 5A). No such effect was revealed for the GA-fixed cells or for the attachment system at 2.8°C (Fig. 5B). A positive cooperative binding was detected in all experiments performed with untreated cells at 37°C. However, the position and width of the region of the function indicating this effect varied. An obvious explanation for this variation is the fluctuation in the status of the cells. From the Scatchard plots the numbers of CRS were estimated, and values ranging between 3,000 and 6,000 CRS per cell were obtained for all three series studied.

The Scatchard plots were further used for calculations of the association constants, and on a per-receptor-site (CRS) basis, a value between 0.8×10^{10} and $1.2 \times 10^{10} \text{ M}^{-1}$ was obtained for the virus-cell interaction at 37°C, whereas for the other two series the constants were in the range of 0.3×10^{10} to $0.4 \times 10^{10} \text{ M}^{-1}$.

DISCUSSION

In this study we have specifically focused on the early interactions occurring between human Ad2 and CRS of the HeLa cell plasma membrane during the phase of virion attachment.

Experiments designed to study the temperature dependence demonstrated that the initial rates of viral attachment were strongly temperature dependent, as previously indicated by Philipson (25) and Inoye and Norrby (12). A shift in the rate increase by a factor of 2 was established for temperatures above 20°C. The reproducibly observed inflection point of the kinetics curve might be due to increased possibilities for the CRS to move laterally in the plasma membrane. This could be a consequence of alterations in the physical status of the lipid matrix, such as gel-to-liquid crystalline phase transitions or phase separations (2) or both. Besides an increased movement, alterations in the lipid moiety would allow a hypothetical unmasking of individual units within the receptor sites, further leading to higher affinity for virus particles. These ideas emerging from temperature dependence experiments are likewise supported by the results obtained with GA-fixed cells. The chemical fixation or cross-linking will lead to an increase in the rigidity of the membranes, where proteins no longer can undergo conformational changes or diffuse freely in the membrane (4, 21, 30). When our data were converted into Arrhenius plots (not shown), the activation energy of virus attachment was shown to be around 42 kJ/mol at temperatures above the inflection point, where-

studied in a fluorescence microscope. (A) cells fixed with GA directly after incubation at 2.8°C; (B to D) cells fixed with GA after transfer to 37°C and a further incubation for 0.5 (B), 5 (C), and 20 (D) min, respectively. All photographs are of the same magnification; bar = 25 μm .

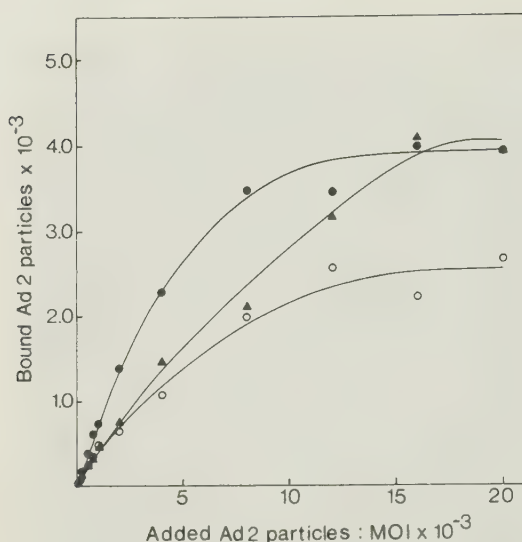


FIG. 4. Multiplicity dependence of the adenovirus attachment. Ad2 particles were added to cells at different MOIs, and the number of attached virions was estimated after different periods of incubation: ●, at 37°C for 45 min; ▲, at 2.8°C for 3 h; and ○, GA-fixed cells at 37°C for 45 min. For details, see the text.

as about 59 kJ/mol was obtained for the curve below this point, again indicating a temperature-dependent alteration of the system. In analogous attachment experiments with human rhinovirus type 2 and poliovirus type 2, linear Arrhenius plots were obtained, with activation energies of around 54 kJ/mol (20). Provided that the cells were pretreated at 0°C for more than 3.5 h, the viral attachment kinetics revealed an inflection point at 18°C. An explanation suggested for this effect was the introduction of the receptor sites into a fluid surrounding, as a consequence of phase separations. In analogy to the above shifts in activation energies at distinct temperatures, comparable effects at around 25°C have been demonstrated for, e.g., lipid analog diffusion in plasma membranes of human fibroblasts (13).

In the experiments with different MOIs, we applied the analysis technique of Scatchard (29) to estimate the number of virus receptor sites per cell and the association constants between viruses and cells at steady-state conditions. The number of CRS for adenoviruses on the HeLa cells was in the range of 3,000 to 6,000, which is in good agreement with previous determinations (26). The number of CRS was not affected by different temperatures of the virus-cell mixtures, and fixation of cells with GA also displayed no or minor effects on CRS numbers. A positive cooperative binding is a well-established phenomenon when multivalent ligands are attached to cells (14, 28), and Scatchard plots for untreated

cells and viruses at 37°C indicate such a cooperative binding at Ad2 attachment. Low temperatures and chemical stabilization by fixation of cells clearly inhibit this effect. In support of our latter finding, it has been reported for other systems that cells fixed with minute amounts of GA do not bind ligands in a cooperative way, as is the case otherwise (32). In addition to the viral multivalency, it is possible that the mechanisms to achieve the demonstrated binding pattern involve either an increase in the affinity of the CRS for viruses or an increase in the number of available binding sites within the CRS. Even though it is pointed out above that the number of CRS is unchanged whether a positive cooperative binding is obtained or not, there is a possibility for unmasking each unit within the CRS, leading to higher affinity of viruses for these sites. The association constants show that cooperative binding is a result of an increased affinity for viruses. The constant for untreated cells at 37°C is two to three times higher than that obtained for cells incubated at 3°C or for GA-fixed cells.

Schlessinger et al. (31) have suggested that the lateral aggregation of membrane components forming the caps and the subsequent immobilization are caused by a ligand-induced conformational change of the receptor molecules or by receptor-ligand complexes that in turn increase the binding affinity between mobile receptors. Our experiments with rhodamine-labeled Ad2 show that adsorbed viruses redistribute in the membrane. Thus, after 10 min at 37°C, about 15% of the cells displayed capping of viruses. Azide, cytochalasin B, and 2-deoxyglucose treatment of cells inhibited the capping, as did low incubation temperatures and prefixation of cells with GA. The methods employed to obtain inhibition of the capping effect have been described for several other model systems (33), and we therefore conclude that the phenomenon studied in our system is a true capping. The observation that only 15% of the cells exhibited caps in our experiments might be explained by the fact that cells with high rates of endocytotic activity have a lower tendency to form caps than do cells with lower degrees of endocytosis (34). Thus, capping might be regarded as a consequence of competition between a lateral aggregation in the membrane and internalization of the ligand. Capping of other nonenveloped animal viruses has been reported for the systems of mengovirus (8), frog virus type 3 (1), reovirus type 3 (6), and also for the mycoplasma-specific bacteriophage L3 (9). Mengoviruses capped on Erlich ascites tumor cells are rapidly internalized (8), and reports concerning other model systems clearly demonstrate that capped ligands are subsequently internalized (33). However,

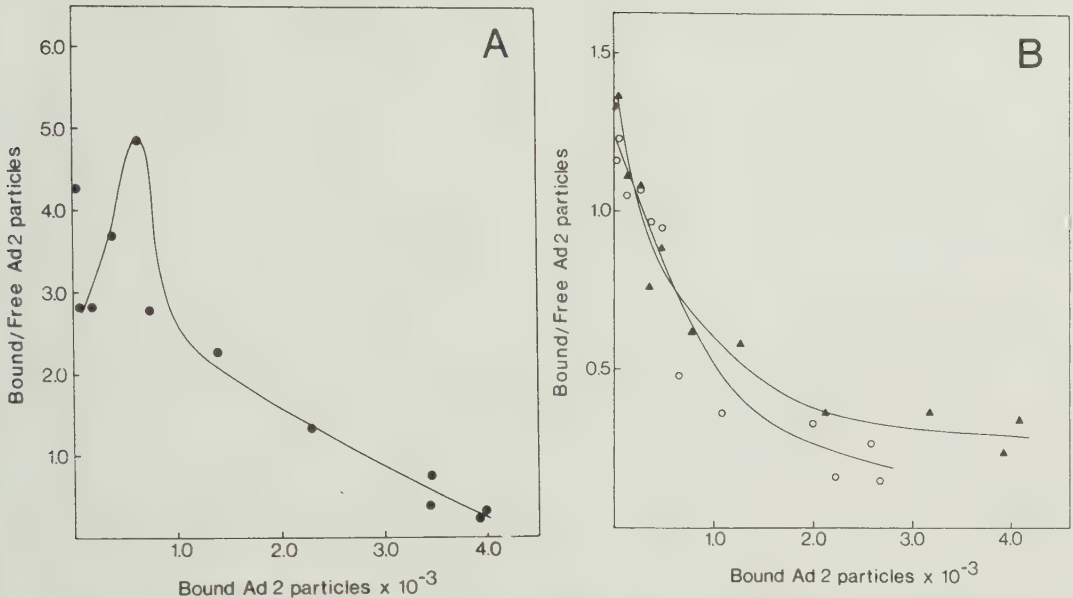


FIG. 5. Scatchard analysis of adenovirus attachment to HeLa cells. The attachment data of Fig. 4 were converted into Scatchard plots. Symbols: ●, virus-cell interaction at 37°C for 45 min; ▲, virus-cell interaction at 2.8°C for 3 h; and ○, virion attachment to GA-fixed cells at 37°C for 45 min. Note the different scales of the ordinates.

the relevance of capping for the following steps of internalization and uncoating in the adenovirus system is at this point speculative, but we are at present studying some aspects of these early events.

In this work, we present suggestive evidence that Ad2 attachment to HeLa cells leads to such alterations in the plasma membrane that redistribution of the viral receptors and possibly configurational changes of the entire receptor sites occur. These concomitant events result in cooperative binding and capping of attached virions.

ACKNOWLEDGMENTS

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Entry of Adenovirus 2 into HeLa Cells

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Adenovirus 2 (Ad2) uncoating was analyzed as the destabilization of virions which renders the parental genome sensitive to DNase treatment. This event demonstrated a strong temperature dependence, and an Arrhenius plot of initial uncoating rates revealed an inflection point at around 16°C. Activation energies of 331 kJ/mol below and 88 kJ/mol above this temperature were obtained for the uncoating process. Penetration of Ad2 through the plasma membrane was completely inhibited by sodium azide, whereas uncoating was only slightly influenced. This indicated that uncoating had already taken place at the outside of the plasma membrane. Incubations of Ad2 with isolated plasma membranes and cell homogenates showed that intact and metabolizing cells were required for uncoating. We further suggest, based on the inhibitory patterns of EDTA, EGTA, dansylcadaverine, and dithiothreitol, that this destabilization of virions follows upon reorganization in the plasma membrane. In the electron microscope the involvement of coated vesicles was shown for the initial uptake of virions, possibly followed by the engagement of acidic vesicles as judged from the effects of lysosomotropic agents on gene expression. The vectorial transport of virions from the plasma membrane to the nucleus was not affected by reagents interfering with the cytoskeletal system. Consequently, we propose that Ad2 virions are internalized by adsorptive endocytosis.

During the past 20 years several investigations have been reported concerning the early interactions between animal viruses and host cells. Each is an important piece in unraveling the question of how animal viruses gain access to host cells. Continuously, the different reports have been brought together in review articles (5, 10, 22, 25).

Carefully conducted investigations have been performed on different enveloped viruses, e.g., Semliki Forest virus, fowl plaque virus, and vesicular stomatitis virus (27-29). These studies show that the virus particles are internalized by adsorptive endocytosis. The envelopes of internalized particles thereafter fuse with membranes of endocytotic vesicles, a mechanism induced by low pH (41). For some members of the enveloped virus families *Herpesviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Poxviridae*, fusion of virion membranes and plasma membranes has been demonstrated as the major mechanism for viral entry (6). Both endocytotic uptake and direct penetration through the plasma membrane have been suggested as internalization routes for nonenveloped viruses (25). It has been stated that reoviruses, once inside the cell, utilize lysosomes to mediate uncoating in the productive cycle (37). For adenoviruses 2 and 5, destabilization in vitro is not achieved in the presence of isolated lysosomal enzymes, implying a nonlysosomal involvement for this process (1).

Adenovirus attachment to specific cellular recognition units leads to redistribution of the cellular recognition units on the cell surface (32, 33). This process has been suggested to facilitate viral internalization (32). The viral capsid is destabilized in close connection with the latter event, leading to a DNase-sensitive viral genome (24, 34, 38). Whether adenoviruses pass through acidic vesicles, before genome expression in the nucleus, is still unknown. However, the engagement of cytoplasmic vesicles has been observed, since adenovirus 2 (Ad2) mediated enhancement of the toxicity of *Pseudomonas* toxin and toxin conjugated to the epidermal-growth factor, by inducing a release of the toxin from intracellular vesicles (12).

In the present investigation we have studied the early

interaction between Ad2 and HeLa cells, by physical and chemical manipulations of the steps connected with internalization of virions. To be certain that we studied the infectious fraction of virus particles, the effect on gene expression was also followed.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenovirus was propagated, radioisotope (3 H)thymidine labeled, and purified as described previously (11).

Attachment studies. The general procedure of virion attachment was as described earlier (39). HeLa cells, washed once in phosphate-buffered saline (PBS) and suspended in PBS at a density of 5×10^7 cells per ml, were infected with [3 H]thymidine-labeled virions (3×10^6 cpm per 10^{12} virions) at a multiplicity of infection (MOI) of 340. The possible effect on the attachment step was studied in the presence of the following reagents: sodium azide (10 to 50 mM), 2-deoxyglucose (10 to 50 mM), sodium azide (10 to 50 mM) together with 2-deoxyglucose (10 to 50 mM), dithiothreitol (DTT) (2 to 10 mM), EDTA (5 to 20 mM), ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) (5 to 20 mM), cytochalasin B (15 to 60 μ g/ml), colchicine (0.1 to 0.4 mM), trifluoperazine (10 to 50 μ M), dansylcadaverine (0.1 to 1.0 mM), ammonium chloride (10 to 40 mM), chloroquine (0.1 to 0.4 mM), and methylamine (10 to 40 mM). Cells were equilibrated 10 or 30 min before virus addition at 37°C with the various reagents, which were maintained during virus attachment. This step was followed for 60 min.

Isolation and radioisotope labeling of antibodies. The immunoglobulin fraction from a serum raised in rabbits against freeze-thawed (FT) Ad2 particles was ammonium sulfate precipitated and affinity purified on an Ad2-AH-Sepharose 4B column. The Ad2-AH-Sepharose was prepared as previously described (39). The purified virion-specific immunoglobulin, anti-FT-Ad2 immunoglobulin G, was 125 I-labeled with 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen), as described by Fraker and Speck (13).

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The specific radioactivity of the labeled immunoglobulin was 1.0×10^8 to 1.9×10^8 cpm/mg of protein.

Virus penetration. Cells were washed once, resuspended in PBS at a concentration of 5×10^7 cells per ml, and subsequently treated with reagents as described above. Virus was added at an MOI of 10,000, and the cell-virus mixtures were incubated at 3°C for 45 min, resulting in ca. 2,500 attached particles per cell. To remove unattached virions, the cells were washed twice and resuspended in PBS at a concentration of 5×10^7 cells per ml, after which the suspensions were incubated at 37°C for 40 min. These incubations were ended by cooling the suspensions to 3°C . Anti-FT-Ad2 [^{125}I]immunoglobulin G was added at a calculated ratio of 2,000 molecules per attached virus particle, and the incubations were continued for 1 h at 3°C . Free immunoglobulin G was removed by two cycles of washing in PBS, and the amounts of immunoglobulin still associated with the cells were determined. The maximum and minimum values of the virus that remained detectable on the outside of the cells were determined from two series of cells with attached virus; one was kept at 3°C throughout the entire incubation procedure, and the other was kept at 37°C . Nonspecific adsorption of anti-FT-Ad2 [^{125}I]immunoglobulin G was determined for mock-infected cells in the presence of the various reagents.

DNase sensitivity of the viral genome. The procedure for DNase sensitivity determination was essentially as described by Joklik (21). Cells were processed as above, and [^3H]thymidine-labeled virus was added at an MOI of 1,000. After attachment for 5 min at 3°C , cells were diluted five times, after which unattached virus was removed by sedimentation of the cells. In temperature experiments infected cells, at a density of 10^7 cells per ml in Eagle minimal essential medium, were further incubated at 3, 10, 15, 20, 25, 27.5, 30, 34, and 37°C . Samples were withdrawn after 1, 5, 10, 20, 40, and 60 min, and cells were sedimented and resuspended at a concentration of 5×10^6 cells per ml in 10 mM sodium phosphate buffer, pH 7.5, containing 10 mM MgCl_2 . The samples were kept on ice until sonicated four times at 15 s each. A volume of 450 μl of each homogenate was treated with 1 mg of DNase I for 30 min at 37°C , and undegraded [^3H]DNA of virions was precipitated with trichloroacetic acid at a final concentration of 10%. The background level of spontaneous viral nucleic acid degradation was measured by direct precipitation of parental DNA without any DNase treatment. Trichloroacetic acid precipitates were pelleted, washed with ethanol, and dissolved in Protosol. Collected supernatants were neutralized with sodium hydroxide, and radioactivity was measured along with dissolved pellets.

To study the effect on viral capsid destabilization of the reagents listed above, these reagents were added and incubated together with cells in PBS at a density of 5×10^7 cells per ml for 30 min at 37°C before virus infection. Virus was added at an MOI of 340 and allowed to attach for 5 min at 37°C . Unattached virions were removed, and the cells were resuspended in PBS supplemented with the appropriate reagents. Samples were withdrawn from incubation mixtures maintained at 37°C for a total of 40 min.

Reagents demonstrating an inhibitory effect on the kinetics of viral nucleic acid DNase sensitivity were analyzed with respect to the reversible nature of the inhibition. Thus, agents were removed after 40 min of incubation at 37°C , and cells were subsequently incubated for 2 h at 37°C . No differences in the levels of maximum DNase sensitivity were obtained when infections were made with multiplicities of

340 and 1,000 particles per cell at 37°C and 3°C , respectively.

In vitro destabilization of adenovirions was analyzed in the presence of isolated plasma membranes (4, 20) or total cell homogenates, the former together with 10 mM concentrations of MgCl_2 , CaCl_2 , or both and of ATP, GTP, or both. Virions were added to the plasma membranes and cell homogenates in calculated quantities yielding MOIs of 24 or 340 virions per cell equivalent. In some experiments incubation mixtures were supplemented with cytoplasmic extracts.

Virus polypeptide production. The production of an early and a late viral antigen, the 72,000-molecular-weight DNA-binding protein (DBP) and the fiber protein, respectively, was analyzed in the presence of the reagents listed above. Cells were pretreated with the various reagents, and virus was added at an MOI of 1,000 and attached for 40 min at 37°C . Reagents and unattached viruses were removed, and cells were diluted to 4.5×10^5 cells per ml in Eagle minimal essential medium containing 5% fetal calf serum and incubated for 30 h. Samples were withdrawn at different times, and cells were sedimented and disrupted in 50 mM Tris-hydrochloride buffer, pH 8.1, containing 1% Triton X-100, for 40 min at room temperature. This treatment was sufficient to give maximum yields of both DBP and fiber protein (data not shown). Cell debris was sedimented at $3,800 \times g$ for 10 min, and supernatants were analyzed for the two viral antigens by rocket immunoelectrophoresis (23) in agarose gels containing monospecific antiserum against each protein.

During the 30-h incubation period, cell samples were assayed for total numbers and viability by the trypan blue exclusion method. No differences were discernible between controls and cells treated with reagents.

Electron microscopy. Cells were harvested, washed with PBS, and incubated in the presence of different reagents as described above. After infection with Ad2 at an MOI of 5,000 and further incubation for 20 min at 37°C , cells were washed, suspended in PBS, and kept at 0°C at a density of 10^6 cells per ml. Fixation was made in 0.015% glutaraldehyde for 30 min at 4°C in a roller bottle as described by Persson et al. (33). After two cycles of washing in PBS, cells were postfixed and dehydrated essentially as described by Ryter and Kellenberger (36) and embedded in Epon.

Protein determination. Protein was determined by the method of Hartree (15) with bovine serum albumin as the standard.

Liquid scintillation spectrometry. Radioactivity measurements in the DNase assay of supernatants and pellets dissolved in Protosol were performed in a cocktail of toluene-methanol (1:1) containing 0.4% Omnifluor. All other radioactive samples were analyzed in Ready-Solv HP/b.

Chemicals. Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories Ltd., Irvine, Scotland. Omnifluor, Protosol, and [^3H]thymidine (75 Ci/mmol) were purchased from New England Nuclear Chemicals GmbH, Dreieich, Federal Republic of Germany. Agarose type I, bovine serum albumin, chloroquine, colchicine, cytochalasin B, 2-deoxyglucose, DNase I (from bovine pancreas), DTT, EGTA, monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemical Co., St. Louis, Mo. Ammonium chloride, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic of Germany. PD-10 columns with Sephadex G-25M and AH-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen) was from Pierce Chemical Co., Rockford, Ill. Triton X-100 (scintillation grade) was from BDH, Poole, England. Ready-

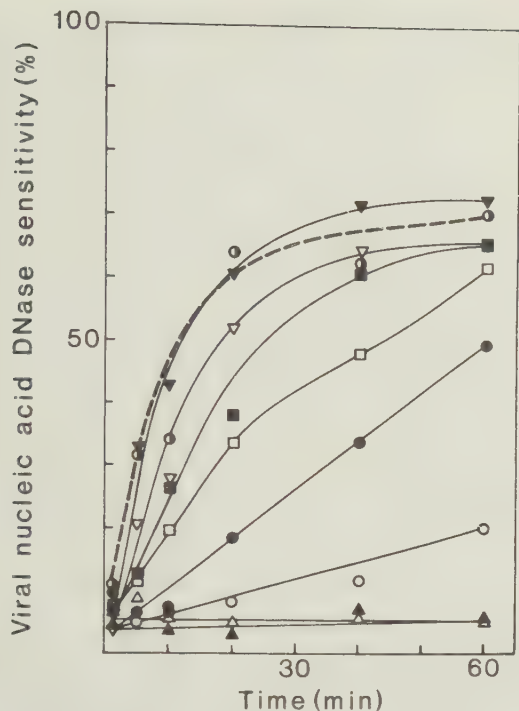


FIG. 1. Temperature dependence of the Ad2-uncoating kinetics. Cells with attached [^3H]thymidine-labeled virus particles (at 4°C for 5 min) were incubated at the indicated temperatures. Samples from each incubation mixture were withdrawn at intervals, and viral uncoating was determined as described in the text. Symbols: Δ , 3°C ; $\blacktriangle$, 10°C ; $\circ$, 15°C ; $\bullet$, 20°C ; $\square$, 25°C ; $\blacksquare$, 27.5°C ; ∇ , 30°C ; $\blacktriangledown$, 34°C ; and $\odot$, 37°C .

Solv HP/b was obtained from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Temperature dependence of Ad2 uncoating. Early in infection, virions of the inoculum were destabilized rendering the parental viral genome sensitive to treatment with exogenously added DNase. We have adopted the word uncoating for this process. The uncoating of cell-attached virions was analyzed at temperatures between 3 and 37°C . Uncoating was strongly temperature dependent (Fig. 1). The initial rates of viral uncoating, at the different temperatures, were calculated from the slopes in Fig. 1. At temperatures below 15°C , uncoating was barely detectable, whereas above 20°C , the uncoating rates increased rapidly to reach a maximum at around 34°C (Fig. 2). The data of Fig. 1 were converted into an Arrhenius plot (inserted in Fig. 2), and a break in the curve at ca. 16°C was obtained. The activation energies for the uncoating process were calculated to 88 kJ/mol above 16°C and 331 kJ/mol below this temperature.

Viral attachment in the presence of various reagents. Before virus internalization and uncoating were studied in the presence of different reagents, these reagents were added to virus-cell mixtures to monitor any possible effects on the attachment step. A concentration of 0.1 mM dansylcadaverine

displayed no effect, whereas concentrations of 0.5 and 1.0 mM resulted in inhibitions of attachment of 12 and 41%, respectively. 2-Deoxyglucose (10 to 50 mM) reduced attachment by ca. 20%, and the combination of sodium azide and 2-deoxyglucose resulted in the same inhibition as did 2-deoxyglucose alone. The influence on attachment of all the other reagents was considered negligible ($<10\%$). The data above is in full accordance with the effects of sodium azide, 2-deoxyglucose, cytochalasin B, and colchicine that have been described previously (33).

Influence of various reagents on viral penetration. Affinity-purified ^{125}I -labeled immunoglobulins, against FT-Ad2 particles, were used to determine the amount of attached Ad2 unable to penetrate the plasma membrane of HeLa cells treated with different reagents. Sodium azide (50 mM) inhibited the penetration of Ad2, indicating a dependence of metabolic energy (Fig. 3A). A reduced concentration of azide (10 mM) still displayed the same severe impairment of penetration. Another metabolic inhibitor, 2-deoxyglucose, did not show any inhibitory effect. EDTA and EGTA both reduced the amount of internalized Ad2 to about 25% as compared with internalization in control cells, pointing out the importance of bivalent cations for this process. The concentrations of EDTA and EGTA could each be decreased to 5 mM without loss of the inhibitory effect. The disulfide-reducing agent DTT, at a concentration of 10 mM, revealed a slight interference with the process of penetration. Dansylcadaverine (1 mM) totally blocked this event, and a dose-response relationship was obtained when concentrations down to 0.1 mM were analyzed. No inhibition was seen when trifluoperazine, an antagonist to calmodulin, was present in the incubation medium. This was also the case for colchicine and cytochalasin B, agents interfering with micro-

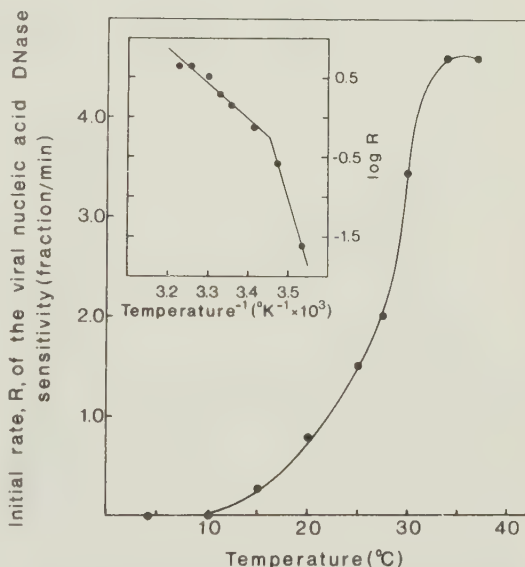


FIG. 2. Temperature dependence of the initial rate of adenoviral uncoating. The initial rates of viral uncoating at the different temperatures were calculated from the slopes in Fig. 1 to give the fraction of uncoated virions per minute. Insert, Conversion of the results into an Arrhenius plot.

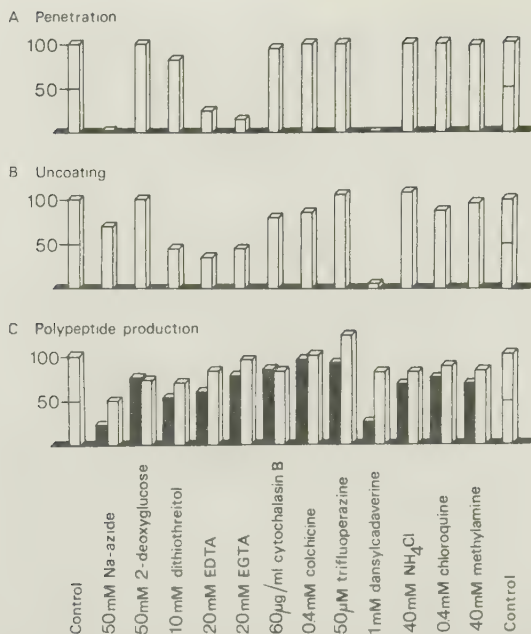


FIG. 3. Influence of added reagents on Ad2 internalization. (A) Penetration of Ad2 through the plasma membrane. ¹²⁵I-labeled antibodies against virus particles were used to follow the events of viral penetration. (B) Uncoating of Ad2, measured as the DNase sensitivity of the viral genome. (C) Synthesis of viral polypeptides. The amounts of DBP (solid bars) produced 7 h postinfection and of fiber antigen (empty bars) produced 13.5 h postinfection were determined by rocket immunoelectrophoresis. Ad2 was added to cells pretreated with the indicated reagents as described in the text. The control levels of the three processes, in normally infected cells, were set to 100%.

filaments and microtubules. None of the analyzed lysosomotropic agents, NH₄Cl, chloroquine, or methylamine, interfered with the step of internalization.

Uncoating in the presence of different reagents. The patterns of chemical interference with uncoating were similar to those obtained for penetration (Fig. 3A and B). One difference was the more pronounced inhibitory effect of DTT, which also displayed a dose-response relationship in the interval of 2 to 10 mM. Also noticeable was the poor effect of azide on the uncoating step, as compared with the significant inhibition of penetration. However, the combination of 50 mM azide and 50 mM 2-deoxyglucose resulted in a complete inhibition of the uncoating (data not shown). A dose-response relationship was obtained for dansylcadaverine at concentrations from 0.1 to 1.0 mM, whereas EDTA and EGTA showed almost the same inhibitory patterns even when the concentrations were decreased to 5 mM. An effect of ca. 20% inhibition was observed when cytochalasin B was present during the incubation of virus with cells.

Reversion of the inhibitory effect of different reagents on viral uncoating. Virus-infected cells were incubated in the presence of reagents, shifted to reagent-free media, and further incubated as described in the legend to Fig. 4. It was conclusively demonstrated that the inhibitions were re-

versed after the reagents were removed, even if these events proceeded slowly for some of the most potent inhibitors.

To determine whether it was possible to overcome the inhibition of uncoating caused by EDTA or EGTA, cells were carefully washed with 5 mM of either of these reagents before they were suspended in PBS containing 5 mM EDTA or EGTA. To each cell suspension was added 10 mM of Mn²⁺, Mg²⁺, or Ca²⁺, and these mixtures were incubated at 37°C for 30 min. Virus addition and further treatments were as described above. It was reproducibly demonstrated that any of the analyzed bivalent cations could reverse the inhibitory effect of both EDTA and EGTA (data not shown).

Uncoating of Ad2 in cell-free extracts. Some of the results indicated that uncoating takes place at the plasma membrane. To attain more information, virus particles were attached to isolated plasma membranes or membranes of cell homogenate. The incubation mixtures were also supplemented with bivalent cations, energy donors in the form of

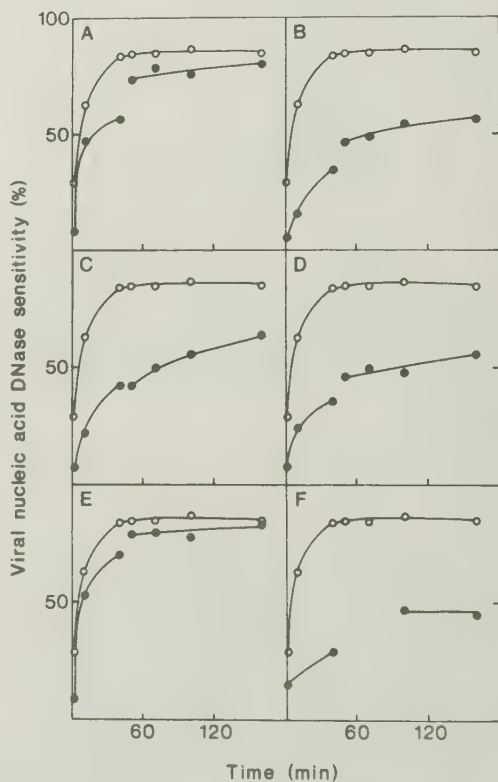


FIG. 4. Reversion of the inhibitory effect of different reagents on viral uncoating. Ad2 was attached for 5 min at 37°C to cells pretreated with indicated reagents. After removal of unattached virions, the infected cells were further incubated for 40 min at 37°C. At this point reagents were removed (gaps in the figures), and cell-virus mixtures were incubated for an additional period of 2 h. Virus uncoating was measured as described in the text. Panels illustrate the situation for (A) 50 mM sodium azide, (B) 10 mM DTT, (C) 20 mM EDTA, (D) 20 mM EGTA, (E) 60 µg of cytochalasin B per ml, and (F) 1 mM dansylcadaverine. Open circles indicate control series, and filled circles indicate reagent-treated series.

ATP or GTP. These incubations showed that none of the combinations analyzed possessed the capacity for a proper uncoating of adenovirus in vitro. This means that either the uncoating does not take place at the plasma membrane or that intact and functional cells are required.

Synthesis of virus-specified proteins. To monitor the indirect effects on gene expression of inhibitors of penetration and uncoating, we studied the production of an early viral antigen, DBP, and a late viral structural antigen, the fiber, in the presence of the previously described set of reagents. The inhibitory effects for most of the reagents were more pronounced for the synthesis of DBP at 6 to 8 h postinfection than for fiber synthesis at 12 to 15 h postinfection (Fig. 3C). It was demonstrated that the reagents which had a negative effect on penetration and uncoating also were efficient in blocking DBP synthesis. In addition, infections in the presence of lysosomotropic agents resulted in a 25 to 30% inhibition. The reagents which displayed a substantial impairment on the polypeptide production were tested at lower concentrations. Thus, 10 mM azide and 0.5 mM dansylcadaverine reduced the DBP production to 50% of the control values, whereas 5 mM DTT had no effect. Reagents which caused a distinctive and reproducible decrease in fiber production were azide, DTT, and 2-deoxyglucose. The lower interference of the majority of the reagents on fiber synthesis, compared with their effect on DBP production, was most probably due to the reversible nature of the inhibition when these reagents were removed, as was also demonstrated in the uncoating assay (Fig. 4). Supporting this idea was the finding that fiber production progressively increased up to control values during a 30-h kinetic study.

In a set of experiments reagents were present during the entire incubation period, to maintain the initial inhibitory effects on internalization. However, for most of the reagents the long exposure was lethal to the cells, as judged by trypan blue exclusion analyses.

Electron microscopy of early interactions between Ad2 and cells. To obtain further information regarding the effects of the reagents used, virus-infected cells were processed for electron microscopy. Virion localization, as revealed by direct visualization, corresponded well with previous data obtained by indirect techniques. Thus, the most potent inhibitors of penetration and uncoating yielded a large proportion of virus left on the cell surface or enclosed within vesicles in the cytoplasmic compartment. In the case of sodium azide, only 1% of the scored Ad2 particles were released as free entities inside the cytoplasm, which should be compared with 82% in the control series (Table 1 and Fig. 5). Also for dansylcadaverine, DTT, and EDTA, the amounts of free virus particles inside the cells were low, 11 to 14%. On the other hand, cells treated with these reagents or cytochalasin B showed relatively higher proportions of virions inside vesicles. It was observed that virions were internalized in vesicles, of which a predominant fraction appeared coated (Fig. 5B). Vesicles containing one virus particle were most frequent, except when cells were treated with DTT, in which case vesicles were noncoated (Fig. 5C).

DISCUSSION

To continue our previous studies on the specific and cooperative binding of Ad2 to HeLa cells, we have, in the present investigation, focused on the subsequent steps of virion internalization. In close connection to the penetration of the plasma membrane, it has been shown by others that the virion capsid is destabilized (24, 34, 38).

The uncoating kinetics of cell-attached Ad2 was strongly

temperature dependent, as previously indicated by Philipson (34) and Lawrence and Ginsberg (24). The maximum level of DNase sensitivity of the parental DNA was also in excellent correspondence with previous results (24, 34). Calculated figures of activation energies for the process of uncoating yielded values in correspondence to reports for membrane processes above and below lipid phase transitions (19) and for established systems of endocytosis (26). A similarly strong temperature dependence for the endocytotic uptake of Semliki Forest virus (27), asialoglycoprotein (40), and the plant toxin ricin (35) has also been reported.

To unravel the mechanisms behind adenovirus internalization, we analyzed the effect of twelve reagents on the steps of viral penetration, uncoating, and early and late gene expression. Under the conditions employed, none of the reagents were deleterious to cells. Our biochemical data were supported by visualization in the electron microscope of the actual cellular distribution of virions. All processes were energy dependent, since they were more or less inhibited by sodium azide. Interestingly, the penetration step was completely blocked, whereas uncoating was inhibited only by 25%, indicating a virion capsid labilization already at the outer surface of the plasma membrane and possibly in close connection with previously stated patching of the viral receptors (32, 33). This finding is analogous to the situation in the nonenveloped picornavirus system, which also begins with a modification of virions already at the cell surface (9). On the other hand, the more complex RNA-viruses of the *Reoviridae* have been reported to undergo a partial uncoating after entry into the cytoplasmic compartment (37). For another nonenveloped virus family, the *Papovaviridae*, studies in the simian virus 40 system have indicated that the nucleus is the major site for virion uncoating (18).

To further characterize the initial step of adenovirus destabilization, we analyzed the capacity of isolated plasma membrane fractions and cell homogenates to mediate this process, in the presence and absence of exogenously added energy donors and bivalent cations. Since the procedure for plasma membrane isolation has been reported to be critical for the labilization of picornaviruses in vitro (9), we employed the two-phase separation system. Our efforts to uncoat adenovirions in vitro turned out negative, thus supporting the previous data of Brown and Burlingham (3) but contradicting the findings of Boulanger and Warocquier (2). However, the important conclusion of these observations is

TABLE 1. Cellular distribution of Ad2 particles in response to different reagents as revealed by electron microscopy^a

Reagent	Relative amount of virus particles (%) ^b		
	On cell surface	Within vesicles	Free in cytoplasm ^c
Control	13	6	82 (65)
50 mM NaN ₃	84	15	1 (0)
50 mM 2-deoxyglucose	42	5	52 (45)
10 mM DTT	63	26	12 (17)
5 mM EDTA	67	19	14 (31)
60 µg of cytochalasin B per ml	57	19	24 (39)
0.4 mM colchicine	40	8	52 (27)
0.5 mM dansylcadaverine	67	20	12 (19)

^a Ad2 was added to cells pretreated with indicated reagents and incubated at 37°C for 20 min, after which cells were processed as described in the text.

^b Relative distribution of 250 counted virions per sample series.

^c Numbers in parentheses represent the percentages of free virus particles in close proximity to nucleopores.

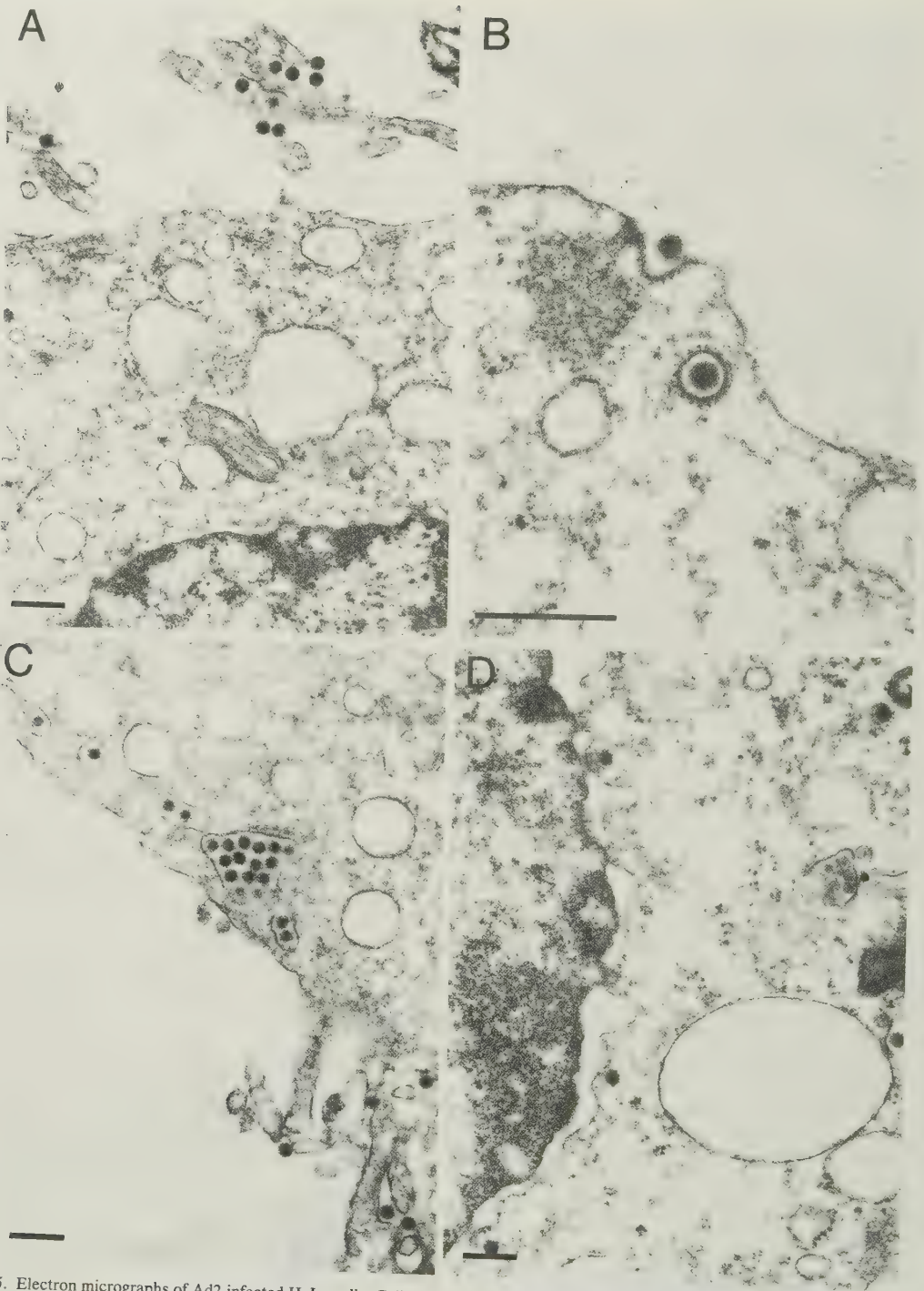


FIG. 5. Electron micrographs of Ad2-infected HeLa cells. Cellular distribution of virions 20 min after infection at 37°C. Panels show cells pretreated with (A) 50 mM sodium azide, (B) 50 mM 2-deoxyglucose, and (C) 10 mM DTT. (D) Control infection without added reagents. Bar, 300 nm.

that intact and metabolizing cells are needed to provide the exact microenvironment for adenovirion uncoating.

Regarding the penetration-uncoating system, a situation opposite to the effect of azide treatment was provoked in the presence of the disulfide-reducing agent DTT. Thus, penetration was close to normal, whereas uncoating was inhibited by ca. 50%. Interestingly, a fourfold increase over the control value of the frequency of virions remaining within cytoplasmic vesicles was observed in the presence of DTT. A possible explanation for this finding could be that internalized but somehow unmodified virions were unable to escape from endocytotic vesicles. It has been reported that DTT inhibits the clustering of enkephalin receptors (16). A corresponding effect can indeed be the case regarding the patching of adenovirus receptors, and this process may be a prerequisite for a proper uncoating.

The steps of penetration and uncoating were further shown to be dependent on the presence of bivalent cations. Since no inhibitory effects or only weak ones were revealed after the addition of trifluoperazine, colchicine, and cytochalasin B, we conclude that the demonstrated cation dependence was not connected with the function of calmodulin or elements of the cytoskeletal system. Dales and Chardonnet (7) showed for the Ad5 system, in the presence of colchicine, a close to normal transport of virions from the plasma membrane region to the nucleus, indicating that intact microtubules are not essential for this migration. This lack of drug effect is also supported by our data on gene expression.

Dansylcadaverine, an established inhibitor of liver and serum transglutaminases (8), has been shown to efficiently block the redistribution of receptor-ligand complexes and subsequent steps of receptor-mediated endocytosis, e.g., of vesicular stomatitis virus and α_2 -macroglobulin (31). In our study, the steps of virion penetration and uncoating were drastically inhibited by this reagent. Therefore, a preceding reorganization of attached virions and an involvement of a bivalent-cation-dependent transglutaminase activity are suggested for uncoating and the adsorptive endocytosis of Ad2.

For the well-characterized system of the enveloped Semliki Forest virus, endocytotic internalization and passage through acid vesicles have been demonstrated (27). In a previous study Boulanger et al. (1) put forward the idea that a lysosomal route might be important for adenovirion destabilization. However, attempts for in vitro uncoating of Ad2 and Ad5 in the presence of lysosomal extracts turned out negative (1). In our study none of the lysosomotropic agents displayed a significant effect on penetration or uncoating, indirectly indicating the lack of acid vesicles in the latter process. This finding is also corroborated by our observation that adenovirions become sensitive to DNase treatment already at the site of the plasma membrane. In the presence of lysosomotropic agents, the synthesis of an early viral antigen (DBP) was reduced by 25 to 30%, whereas the production of the late fiber antigen was inhibited by ca. 15%. These results and the reversible nature of inhibition indicate that virus particles might be transported through acidic vesicles before genome expression takes place in the nucleus. It has also been demonstrated by Ohkuma and Poole (30) and described for the Semliki Forest virus infection (17) that the effect of lysosomotropic reagents is rapidly reversed upon removal. To continue this investigation it would be of interest to analyze an event immediately following a presumptive viral passage through an acidic vesicle.

In the present investigation we have tried to survey the mechanisms of Ad2 internalization. From previous and present results we suggest the following flow of events. Ad2

virions are attached to specific receptors on the plasma membrane of HeLa cells (39). The receptors are subsequently redistributed in the membrane, leading to a clustering of attached virions (33). This reorganization mediates a destabilization of the virus particles leading to a DNase-sensitive viral genome. The modified virions are internalized via vesicles, probably coated, which subsequently develop into endosomes. Uncoating might be a prerequisite for virions to escape from these intracellular vesicles, on the route to the nucleus. During this transport of Ad2 from the cell surface to the nucleus, elements of the cytoskeleton do not seem to be involved. In summary, we interpret our data as favoring a model of adsorptive uptake of Ad2, which resembles the process of receptor-mediated endocytosis defined by Goldstein et al. (14).

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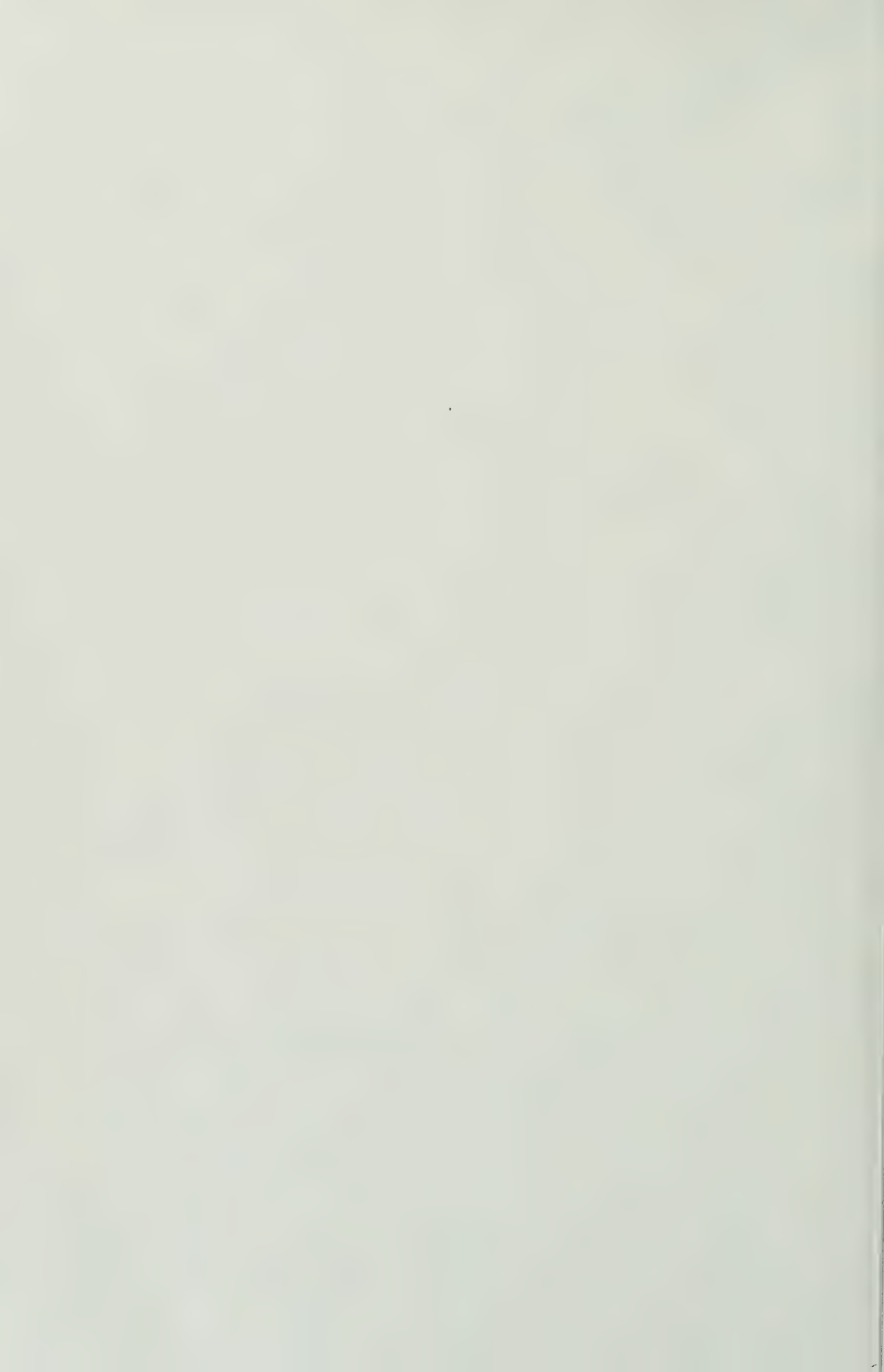
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SIGNIFICANCE OF VESICLES DURING ADENOVIRUS 2 INTERNALIZATION
INTO HeLa CELLS.

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ABSTRACT

In this investigation the early period of adenovirus (Ad2) - HeLa cell interaction has been analyzed by electron microscopy and by biochemical techniques. The steps involved during this period include the disappearance of virions from the cell surface to their subsequent association with the cell nucleus. A destabilization of the virions attached to the intact cell was a necessity for virions to escape from intracellular vesicles. A strong temperature dependence and a rapid escape from a vesicular compartment was demonstrated in temporal kinetic experiments. These vesicles appeared to be acidic since lysosomotropic agents partly inhibited the release of virions from vesicles.

Binding studies of Ad2 to cells in buffers of different pH suggested that adenovirus could bind to cells by two different mechanisms. At low pH the binding was most probably mediated by the penton base and at neutral pH by the fiber protein. The number of receptor sites per cell was 25,000 and 6,000 at low and neutral pH, respectively. It is suggested that the low pH affinity between the penton base and a vesicular membrane is important inside acid vesicles when Ad2 quickly enters the cytoplasm. However, a significant fraction of the virions was possibly internalized by a pathway not requiring a passage through such vesicles.

INTRODUCTION

The early interaction between animal viruses and host cells has been the subject of several investigations during the past 20 years. The conclusions of these investigations are continuously brought together in review articles (3,5,7,18,22). Thus, many viruses are internalized by a pathway referred to as receptor-mediated endocytosis (3,7), which also is utilized by e.g. hormones and plasma proteins (13). This process of internalization has been defined by Goldstein et al. (13). It commences with the attachment of ligands to specific receptor sites on the cell surface; the receptor-ligand complexes are accumulated in coated pits and are subsequently seen inside coated vesicles and endosomes. From the endosomes the ligands are further released into the cytoplasm or transported to different compartments within the cell such as the lysosomes or the nucleus.

Receptor-mediated endocytosis has been suggested as one mechanism of Ad2 internalization (11,37). Thus, it is well established that Ad2 attaches to specific saturable proteins on the plasma membrane of HeLa cells (31,38). The virions are internalized via coated pits and coated vesicles, and are subsequently observed within endosomes (4,11,37). The importance of an acidic pH inside vesicles has been shown for the Ad2-mediated enhancement of the toxicity of *Pseudomonas* toxin conjugated to epidermal growth factor (EGF) (11), and has also been indicated for the sole Ad2 infection of cells (37). The majority of Ad2 virions leaves the endosomes (37). However, the mechanism of this event is unknown.

The intention of the present investigation was to further characterize the prerequisites needed for Ad2 to leave an endocytotic vesicle and also to delineate some of the other steps involved during the journey of adenovirus from the plasma membrane to the cell nucleus.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Ad2 was propagated, radioisotope (^3H -thymidine) labeled, and purified as described previously (9).

Electron microscopy (EM)

Cells were sedimented, washed once and resuspended in phosphate buffered saline (PBS) at a concentration of 5×10^7 cells per ml. Chemicals were added, as specified, and the cells were incubated under slow agitation for 30 min at 37°C . After equilibration for 5 min at 3°C , Ad2 was added at a multiplicity of infection (MOI) of 4,000 particles per cell and allowed to attach for 30 min. Unattached viruses were removed by sedimentation of the cells, which were resuspended in PBS containing the appropriate reagent. Samples were incubated at 37°C for 60 min, at 37°C for 10, 20, 40 and 60 min, or at different temperatures for 60 min in the chemical, kinetic and temperature series, respectively. After the appropriate incubations the cells were washed, suspended in PBS, and kept at 3°C at a density of 1×10^6 cells per ml. Fixation of cells was performed in the presence of 2.5% glutaraldehyde (GA) under slow agitation at 3°C for 30 min. After three cycles of washing in PBS, cells were postfixed and dehydrated essentially as described by Ryter and Kellenberger (34) and subsequently embedded in Epon.

Isolation of nuclei

The kinetics of virus particle transport to the cell nucleus and the possible effect of different reagents and temperatures on this process were studied. Cells were sedimented, washed with PBS, and incubated in the presence of different reagents as described above. The cells were equilibrated at 3°C for 5 min and ³H-thymidine-labeled Ad2 was added at a MOI of 340. Virus particles were allowed to attach for 30 min at 3°C. Unattached virions were removed and the cells were resuspended in PBS, at a concentration of 1×10^7 cells per ml, containing the appropriate reagent. The cells with attached virus were incubated at 37°C for 45 min, samples were withdrawn at intervals and kept on ice. In all experiments the cells were subsequently pelleted and the nuclei were isolated essentially as described by Penman (28). The cells were swelled in 10 mM Tris-hydrochloride buffer, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂ (RSB) and disrupted by addition of Triton X-100 at a final concentration of 0.5% and shaken on a Vortex mixer for 20 sec. The nuclei were sedimented and washed by shaking for 10 sec in RSB containing 1% Triton X-100 and 0.5% sodium desoxycholate (DOC). After sedimentation of the nuclei the nuclear wash was collected together with the first supernatant comprising the cytoplasmic fraction. The nuclear pellet was dissolved in Protosol and radioactivity in the nuclear and the extra nuclear fraction was measured. For each series a background value for non-specific nuclear association of radioactivity was obtained after one min of incubation. These values were subtracted from the amount of radioactivity located in the nuclear fraction of all subsequent samples. Phase contrast microscopic examination showed intact nuclei without contaminating whole cells or membrane fragments; however, the nuclei had a tendency to aggregate as mentioned by Penman (28).

GA-fixation of cells and viruses

HeLa cells were washed and fixed with 0.015% GA as previously described (29). Purified Ad2 was dialyzed over night against 0.1M sodium phosphate buffer, pH 7.3. The virus particles, at a concentration of 2 OD per ml and corresponding to ca. 2×10^{12} particles per ml (24), were fixed with 0.1% GA at 3°C over night under slow agitation. The particles were separated from free GA by gel filtration on a PD-10 column equilibrated in PBS. Neutralized ethanolamine was added to the virus at a final concentration of 1M. The samples were incubated at 3°C for 40 min under slow agitation, whereafter Ad2 was reisolated on a CsCl cushion (10). Virus recovery was 50% and the aldehyde stabilized virions attached to cells with 60% of the efficiency of untreated virions.

DNase sensitivity of the viral genome

The procedure for determination of DNase sensitivity was as described by Joklik (17) and modified by Svensson and Persson (37) except that the cells were disrupted by 0.5% DOC (20).

Radioisotope labeled virus structural proteins

Virus structural proteins, obtained from an Ad2 infected cell culture labeled 15 h p.i. with ^{35}S -methionine, were isolated by DEAE-cellulose chromatography (42).

Attachment studies

HeLa cells were washed once in PBS, once in the appropriate buffer, and finally suspended in the latter buffer at a density of 5×10^7 cells per ml. ^3H -thymidine-labeled virus at a MOI of 340 or

^{35}S -methionine-labeled viral structural proteins at a ratio of 100,000 molecules per cell, were added to the cells and attached at 37°C for 45 and 30 min, respectively. After incubation the samples were diluted five times and the cells were pelleted. The percentage of attached virus or viral structural proteins was calculated after radioactivity measurements. In one experimental series of different buffers, Ad2 was attached to cells pretreated at 3°C with 250,000 fiber molecules per cell for 120 min at pH 7.0. In another experimental series different MOIs of Ad2 were added to cells to determine the number of cellular receptor sites (CRS) at pH 4.8 and 7.0. The buffer systems used were 6 mM universal buffer, pH 3.5-8.5 (2), 10 mM sodium acetate buffer, pH 3.5-5.5, 10 mM sodium phosphate buffer, pH 5.5-8.0 and 10 mM glycine-sodium hydroxide, pH 8.5-9.0, all containing 150 mM NaCl. The pH-values of the different buffers indicated in the figures were obtained in the presence of cells.

Preparation of pentonless virions

Pentonless virions were prepared by dialysis against double distilled water as described by Laver et al. (19). Viruses without pentons were separated from released material by centrifugation at $50,000 \times g$ for 30 min (32). The concentration of pentonless virus was estimated by UV-absorption at 280 nm and calculated as described (24).

The hydrophobicity of pentonless and intact virus was analyzed by incubation for 30 min at room temperature of 1×10^{12} particles with 0.05% Triton X-100 containing 30 μCi ^3H -Triton X-100, in a total volume of 250 μl . The particles were separated from free detergent in linear sucrose gradients (10 - 25% w/v in 50 mM Tris-

hydrochloride, pH 7.5), formed on a cushion of CsCl ($\rho = 1.45 \text{ g/ml}$). The gradients were centrifuged at $100,000 \times g$ for 30 min and fractionated by puncturing.

Protein determination

Protein was determined by the method of Hartree (15) with bovine serum albumin as the standard.

Liquid scintillation spectrometry

Radioactivity measurements in the nuclear isolation and DNase sensitivity assays were performed in a cocktail of toluene-methanol (1:1) containing 0.4% Omnifluor. All other radioactive samples were analyzed in Ready Solv HP/b.

Chemicals

Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories Ltd., Irvine, Scotland. ^{35}S -methionine ($>800 \text{ Ci/mmol}$) was from Amersham Int. Ltd. Buckinghamshire, England. Omnifluor, Protosol, ^3H -thymidine (75 Ci/mmol), and ^3H -Triton X-100 (1.58 mCi/mg) were purchased from New England Nuclear Chemicals GmbH, Dreieich, Federal Republic of Germany. Bovine serum albumin, chloroquine, colchicine, cytochalasin B, 2-deoxyglucose, DNase I (from bovine pancreas), DTT, EGTA, monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemical Co., St. Louis, Mo. Ammonium chloride, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic Germany. PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Triton X-100 (scintillation grade) and DOC were from BDH, Poole, England. Ready-Solv HP/b was obtained from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Kinetics of virus internalization

In this report the term internalization will be used to describe the continuous flow of events from the point of virus disappearance from the cell surface, to the association of the viral nucleic acid with the cell nucleus.

EM was used to measure the frequency of virus particles in various compartments of the cell at intervals from the early attachment to one hour post-infection (p.i.). Virions disappeared from the cell surface within 20 min (Fig. 1). During the first hour of incubation a small fraction of virus particles was seen inside vesicles at the observation times selected. However, the transfer of virions through vesicles was rapid, because already 10 - 20 min after attachment the majority of the virus particles was free inside the cytoplasmic compartment of the cell. The number of virions near the nucleus and in most cases adjacent to clearly visible nuclear pores, increased continuously during the first 30 min, and reached 50% after 45 min (Fig. 1).

The vesicles which contained virus particles were almost exclusively small vesicles (size 100 - 200 nm) and located near the plasma membrane. Some vesicles were coated. It cannot be excluded that a small fraction of the scored vesicles actually were invaginations of the plasma membrane (Fig. 4A).

When nuclei from ^3H -thymidine-labeled virus-infected cells were prepared, the amount of viral nucleic acid inside the nucleus reached a maximum level of 50% after 60 min (not shown). At this time this corresponded to the amount of free virions near the nucleus as judged from the EM studies (Fig. 1). The amount of viral nucleic acid in

the cell nucleus did not further increase during a 5 hour incubation period (not shown).

Temperature dependence of Ad2 internalization

Based on the outcome of the kinetic EM studies of viral localization within the cells, a 60 min incubation period was chosen for the temperature and chemical reagent analyses. The distribution of virus particles between various compartments of the cell at different temperatures was studied by EM. Fig. 2A shows that a temperature above 15°C was required for virus to disappear from the cell surface. Virions did not accumulate inside vesicles at any of the studied temperatures. At 17°C around 6% of the scored virions were free in the cytoplasm. The amount of free virions in the cytoplasm reached a maximum at 25°C and the number of free virions near the nucleus continuously increased up to 37°C (Fig. 2A). The temperature dependence of virus transport to the nuclear fraction was also studied biochemically by means of nuclear isolations (Fig. 2B). These studies revealed a similar pattern of the temperature dependence of the virion transport to the nucleus as was obtained by EM. Thus, both experimental procedures clearly demonstrated that a temperature above 20°C was required to allow a substantial fraction of virions to reach the nucleus.

Influence of various reagents on viral internalization

Internalization in the presence of twelve different reagents was analyzed, by EM at 60 min p.i. and by the nuclear isolation technique at 45 min p.i.

The EM technique. The effect of some of the reagents on virus internalization after 20 min of virus-cell incubation at 37°C have

previously been studied (37). However, in those studies the virus infections were not synchronized and the present kinetic investigation demonstrated that 60 min of incubation was an optimal time to choose. Fig. 3 shows that the inhibitor of oxidative phosphorylation, sodium azide, nearly completely prevented the internalization of Ad2, and consequently 80% of the virions was left in association with the plasma membrane and no virions at all reached the nucleus. In the presence of dithiothreitol 32% of the virions could be seen inside large vesicles. Such vesicles, referred to as multivesicular bodies (MVB), had a diameter of 300-700 nm and usually contained smaller vesicles of different size (Fig. 4B). These structures could be located anywhere in the cytoplasm and appeared to contain one to five virions. In total only 30% of the virions became free in the cytoplasm and associated with the nucleus when DTT was present, as compared with the total level of ca 80% in the control series. In the presence of EDTA and EGTA the majority of the virions was left on the cell surface. When dansylcadaverine was used 44% of the particles could be seen inside vesicles with a small diameter of 100-200 nm and located in the vicinity of the plasma membrane (Fig. 3B). Colchicine, which interferes with microtubular polymerization, almost fully inhibited the transport of free virus particles to the nucleus as judged from the EM analyses. In this case virions appeared to accumulate free in the cytoplasm (Fig. 3C). In the presence of lysosomotropic agents, which previously have been shown to only slightly interfere with the virus early gene expression (37), 15-30% of the virions remained inside vesicles, as compared with the ca 10% level in the control series (Fig. 3). When lysosomotropic agents were employed around half of the virus-containing vesicles were MVB, whereas virions inside MVB were extremely rare in the control

series. In the latter series 50% of the virions reached the nucleus, but in the presence of the most potent lysosomotropic agent, ammoniumchloride, only 15% reached this compartment.

The nuclear isolation procedure. The pattern of reagent-interference with virion transport to the cell nucleus closely resembled the results from the EM-studies. However, in the presence of colchicine ca 30% of the nucleic acid labeled material appeared to reach the nucleus (Fig. 3E).

The effect of DTT and GA-fixation on viral uncoating

Uncoating of Ad2 is believed to take place on the outside of the plasma membrane of the intact cell, and DTT has previously been shown to inhibit this process by 50-60% when compared with the control situation (37). It was therefore of interest to know if this inhibition was due to an effect on the virions or to an interference with the plasma membrane (16). It was clearly shown that only when cells were preincubated with DTT and DTT was present during the virus-cell incubation, the DNase sensitivity was maximally reduced. Incubation of virions alone with DTT did not influence the DNase sensitivity when virions subsequently were incubated with cells in the absence of DTT (Table 1). Virions stabilized by GA-fixation were totally inert to the cell-mediated destabilization process. A likewise lack of destabilization was obtained when the cells were GA-fixed and subsequently exposed to untreated virions (not shown).

Internalization of GA-fixed Ad2

Since it was shown that DTT exerted its function on the cell and significantly impaired the uncoating process but allowed endocytosis, it was of interest to learn how virions behaved which were rendered insensitive to the destabilization process by treatment with GA.

The EM-studies revealed that the GA-fixed virions disappeared from the cell surface at the same frequency as control virions. All virions that were internalized appeared to be trapped inside vesicles, the majority of which being MVB (Fig. 3B and 4B). A low percentage of virions was located inside electron dense vesicles, probably lysosomes. GA-fixed virions were not able to reach the nuclear compartment as demonstrated both in the EM and by the nuclear preparation method (Fig. 3D and 3E).

Attachment of Ad2 and viral structural proteins at different pH

The pH-dependent attachment of Ad2 to HeLa cells displayed two noticeable optima, one at pH 4.7-5.0 and another at pH 6.0-6.5 (Fig. 5). Different overlapping buffer systems were employed and the data suggested that the attachment process was only affected by the change in pH and not by the actual buffering ions. Attachment of the fiber protein was optimal in the pH range of 6.0-6.7, whereas attachment of the penton base drastically increased in the range of pH 4.7-5.1. The hexon attachment was slightly enhanced below pH 5.5 (Fig. 5). Preincubation of cells with fiber at pH 7.0 inhibited a subsequent viral attachment at a pH above 5.2 (Fig. 5). It was further shown that fibers attached at pH 7.0, were released upon acidification below pH 5.2. Under the same experimental conditions it was also shown that virions were not released at any pH after attachment. Cells preincubated at pH 4.8 did not allow attachment of virions at a pH higher than 5.5, indicating that the attachment site for virions under physiological conditions was destroyed at the lower pH (not shown). It was possible to saturate the cellular binding sites at both pH 7.0 and 4.8 when Ad2 was attached to cells at different MOIs. Binding data transformed into Scatchard plots

revealed 6,000 and 25,000 receptor sites per cell at pH 7.0 and 4.8, respectively (not shown). In a control series all the structural proteins and the virions were exposed to the different buffers to learn if any of the ligands precipitated. No such pH-dependent precipitation was discernible and none of the buffers rendered the viral genome sensitive to DNase treatment (not shown).

Hydrophobicity of intact and pentonless virus particles

The possible difference in hydrophobicity between pentonless and intact virus was studied. Pentonless virions were obtained by dialysis against bidistilled water, and SDS-polyacrylamide gel electrophoresis (23) showed that the particles quantitatively lost the antigens of the vertex region (not shown). ^3H -Triton X-100 binding was used to estimate the level of hydrophobicity. The ratio of the amount of ^3H -Triton X-100 bound to intact virions and pentonless particles was 5.4:1, which indicated that intact virions were significantly more hydrophobic than pentonless virions.

DISCUSSION

In this study, which is an extension of a previous investigation on the mechanisms of Ad2 entry into cells (37), the further steps necessary for Ad2 to reach the cell nucleus were examined.

It was demonstrated that virions disappeared from the cell surface very rapidly at 37°C. The maximum level of 50% for nuclear membrane association of virions and parental viral DNA confinement to the nucleus was obtained at 45 and 60 min post attachment, respectively. These observations are in good agreement with previous data on Ad2 and Ad5 transport to the cell nucleus (4,21). Chardonnet and Dales (4) found that the amount of Ad5 in vacuols arrive at a maximum at 10 min after attachment. For Ad2 this process seemed to be less synchronized i.e. ca 15% of the virions was seen in vesicles at all times analyzed within a period of one hour after attachment.

The internalization of Ad2 was strongly temperature dependent as has also been shown for the endocytotic uptake of Semliki Forest virus (SFV) (25), asialoglycoprotein (41), and plant toxin (33). At 15°C no virions appeared free in the cytoplasm but already at 17°C a few percent escaped into the cytoplasmic compartment. A similar temperature dependence has previously been shown for the destabilization of virions on the cell surface (37). Thus, an inflection point in an Arrhenius plot was obtained, for the destabilization process at 16°C. The present study clearly showed that it was necessary for virions to become destabilized on the plasma membrane if they should subsequently escape from endocytotic vesicles (see below). Chardonnet and Dales (4) found that Ad5 was retarded on the cell surface or in vacuols at 25°C. Already at this temperature the majority of Ad2 particles was free in the cytoplasmic compartment. This indicates that the mechanism of

internalization for the two viruses belonging to the same subgroup of adenoviridae not necessarily is identical.

In a previous investigation, the effect of twelve reagents on the early steps of viral interaction with the plasma membrane and on viral polypeptide production was studied (37). These same reagents were used to obtain more information about the steps taking place after virus had left the cell surface but preceding the entry of the viral nucleic acid into the cell nucleus. Several observations of the previous and present investigations are in good agreement. However, a reversion of inhibition was a problem when the polypeptide production was measured (37). This problem was eliminated when the transport to the nucleus was analyzed, since chemicals were allowed to remain during the total period of the study. Thus, all the reagents employed had a more pronounced effect on this event than on the polypeptide production. The inhibition of the nuclear association was at the most 70% of the control level when lysosomotropic agents were used. This indicated that a maximum of around 70% of all virions, predestined to reach the nucleus, was dependent on vesicular structures at low pH. Favouring this idea Seth et al. (36), in a recent investigation, demonstrated that lysosomotropic agents completely blocked the Ad2-enhanced toxicity of a EGF-*Pseudomonas* exotoxin conjugate.

When colchicine was present during infection a puzzling result emerged from the EM-studies as compared with the data obtained by the nuclear isolation procedure. Thus, around 2% and 30% of the virions reached the nucleus, as judged by the former and latter procedures, respectively. In cells treated with colchicine, Dales and Chardonnet (6) previously demonstrated that virions appeared to be retarded on the route to the nucleus due to association with so called annulate lamellae. In an earlier study we showed that colchicine displayed no effect on the synthesis of early or late

virus specified proteins (37). These contradictory results together with the present EM data may be explained by one or more of the following interpretations: i) possibly noninfectious particles were preferentially trapped in the cytoplasmic compartment, or ii) maybe the destabilized virions released the DNA or core-structures, which subsequently managed to reach the nucleus or, iii) any of the techniques employed might produce artifactual results in the presence of colchicine.

When virion destabilization was blocked after treatment of cells with DTT, or when GA-fixed virions were employed the majority of the virus particles was trapped inside MVB, but some could also be seen within more dense vacuols - possibly lysosomes. In cells pretreated with DTT one third of the virions became free in the cytoplasm as compared to 80% in the control series. These values corresponded very well with the 36 and 76% of the virions which became DNase sensitive in the DTT and control series, respectively (Table 1). These findings indicated that a virion destabilization was a prerequisite for the virus to merge from vesicles into the cytoplasmic compartment.

It has been shown that membranes of cell endosomes closely resemble the plasma membrane (27), and further that the pH within an endosome varies between pH 4.8 and 5.5 (39,40). Taken these facts into account intact HeLa cells were used as an "inverted model system" for an endosome. Thus, cells were incubated in different buffers with final pH values ranging between 4.7 and 7.2. The pH-dependent attachment studies revealed two pH-regions at which the attachment was enhanced; one between pH 4.7 and 5.1 and another at ca pH 6.5. From the present investigation it became apparent that virion attachment at low pH was mediated by the penton base, and at a more physiological pH the fiber antigen was

the structure responsible for attachment. Supporting the role of the penton base in virion association with membraneous structures at low pH, Seth et al. (35) demonstrated that anti-penton base-antibodies efficiently inhibited the Ad2-enhanced toxicity of *Pseudomonas* exotoxin conjugated to EGF. The penton base mediates the established cytopathic effect (CPE) (30) and also appeared to affect the total degree of hydrophobicity of the virion. In this laboratory we have recently demonstrated that the Ad2 infectivity will not be neutralized to an extent greater than 50% with anti-penton base-antibodies (C. Wohlfart, unpublished). This finding together with the fact that the effect of lysosomotropic agents never inhibited virion transport to the nucleus by more than 70%, suggests the existence of alternative ways for the Ad2 internalization. One of these being the mechanism of receptor mediated endocytosis coupled to a passage through acidic vesicles.

The fiber antigen is recognized as the viral attachment protein in the adenovirus system (31), and it attaches well to cells under physiological conditions. Fiber structures attached to cells under such conditions are readily detached at a pH below 5.2. Likewise preincubation of cells at an acidic pH demonstrated that the cellular receptor site for this protein was destroyed. Ligand-receptor dissociations at low pH are well-known phenomena in many systems of receptor-mediated endocytosis (1,14,26). In the case of EGF- and asialoglycoprotein-receptor dissociation, this is believed to be due to conformational changes of the receptor site (8).

For the Ad2 system the number of binding sites per cell at pH 4.8 was estimated to ca 25,000, whereas at a physiological pH of 7.0 this number amounted to ca 6,000. These results suggest that the structure(s) recognized by the virus at low pH is not the same as under neutral pH conditions. It has been proposed that SFV is attached

to a plasma membrane protein at a neutral pH, but also reveal affinity for membrane lipids in an acidic environment (12).

In a previous communication we have suggested that Ad2 is internalized by receptor mediated endocytosis (37). Based on the present investigation it is appropriate to supplement this view with the following events: i) A destabilization of the virions rendering the virus genome sensitive to DNase-treatment is an absolute prerequisite for virions to escape from intracellular vesicles. ii) It is also obvious that a rapid passage through acidic vesicles is included in one route for Ad2 internalization. iii) Inside the acid vesicles the affinity between the penton base and the membrane seems important for virions to leave these organelles. iv) It is possible that a significant fraction of the virions is internalized by a pathway where a low pH inside a vesicle and an interaction between the penton base and the vesicular membrane is not required.

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Table 1

DNase sensitivity of the Ad2 genome after 40 min incubation with cells at 37°C a)

Cells preincubated with DTT	Virus preincubated with DTT	DTT present during the virus-cell incubation	DNase sensitivity (%)
-	-	-	76
+	-	+	36
-	+	+	60
-	+	-	77

a) All preincubations were performed at 37°C for 30 min. The DNase sensitivity analysis was as described in the text.

LEGENDS TO FIGURES

Figure 1

Temporal distribution of Ad2 inside cells. Cells with virions attached at 3⁰C were shifted to 37⁰C, and samples were continuously withdrawn and studied in the EM as described in the text. The frequency of Ad2 in different compartments of the cell was scored.

Symbols; ● , Ad2 at the cell surface; ○ , inside vesicles; ▲ , free in the cytoplasm; △ , free near the nucleus.

Figure 2

Temperature dependence of Ad2 compartmentalization within the cell. Cells with virions attached at 3⁰C were further incubated at various temperatures and subsequently studied in the EM or by the nuclear isolation technique as described in the text. The frequency of Ad2 in different compartments of the cell was assessed by the EM technique after 60 min of incubation (panel A). Symbols; ● , Ad2 at the cell surface; ○ , inside vesicles; ▲ , free in the cytoplasm; △ , free near the nucleus. The frequency was monitored of Ad2 ³H-thymidine-labeled parental nucleic acid association with the cell nucleus as revealed by the nuclear isolation technique after 45 min of incubation (panel B).

Figure 3

Influence of added reagents on the cellular distribution of Ad2 particles as revealed in the EM and by the nuclear isolation procedure. Ad2 was attached for 30 min at 3⁰C to cells which were pre-treated with the indicated reagents as described in the text. Cells with attached virions were subsequently incubated at 37⁰C for 60 and 45 min in the EM (A-D) and the nuclear isolation series (E), respectively.

- a) The relative distribution of ca. 150 counted virions per sample series was calculated.
- b) The amount of radioactive parental DNA associated with the cell nucleus, relative to the totally attached Ad2.

Figure 4

Electron micrographs of Ad2-infected HeLa cells. Cellular distribution of virions 60 min post attachment. Panel A: Incubation at 17⁰C; Panel B: Incubation with GA-fixed virions at 37⁰C. Bar 200 nm.

Figure 5

pH-dependence of Ad2 and structural protein adsorption. Cells were incubated with Ad2 or virus structural proteins at the indicated pH-values. The amount of cell-adsorbed virions and structural proteins was measured after 45 and 30 min, respectively. Symbols indicate adsorption of: ● , Ad2; ○ , fiber protein; ▲ , hexon protein; △ , penton base protein; ■ , adsorption of Ad2 to cells pretreated with fiber protein at pH 7.0.

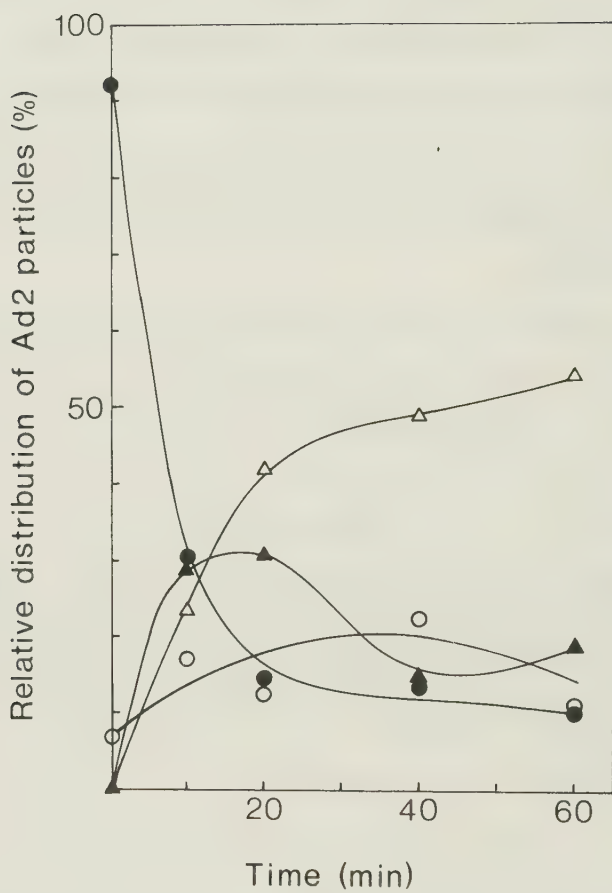


Figure 1

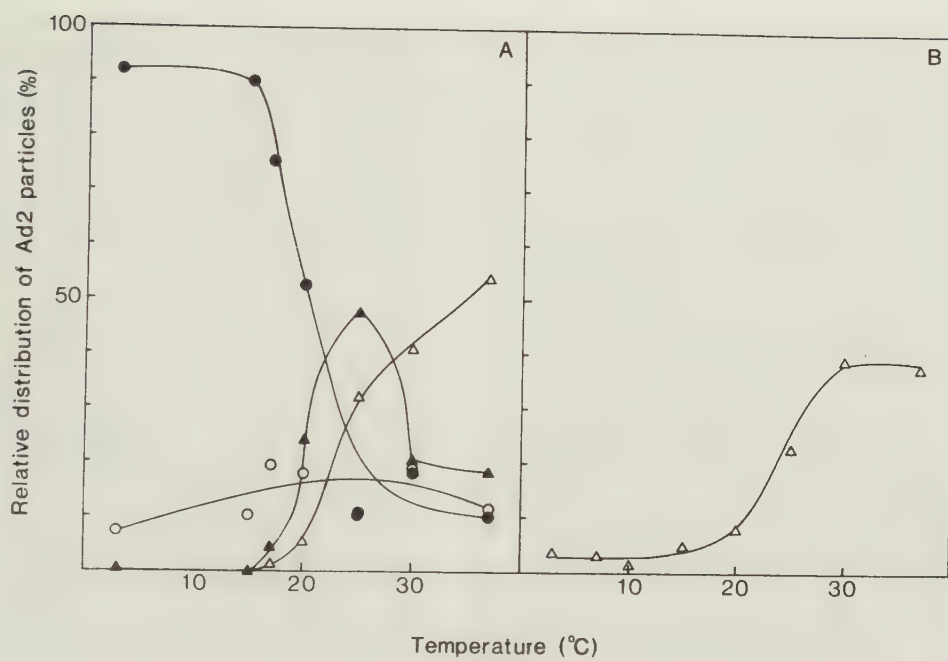


Figure 2

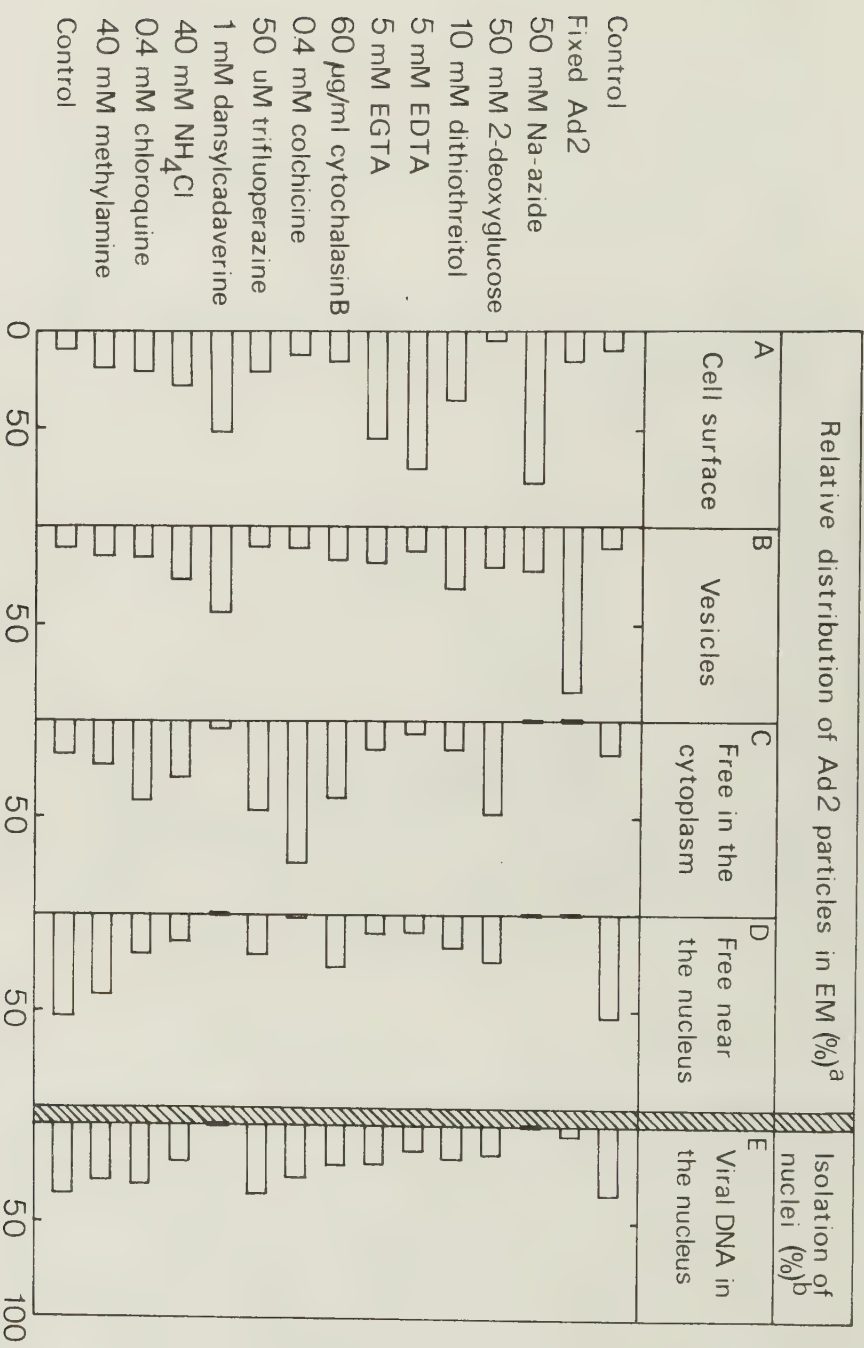


Figure 3

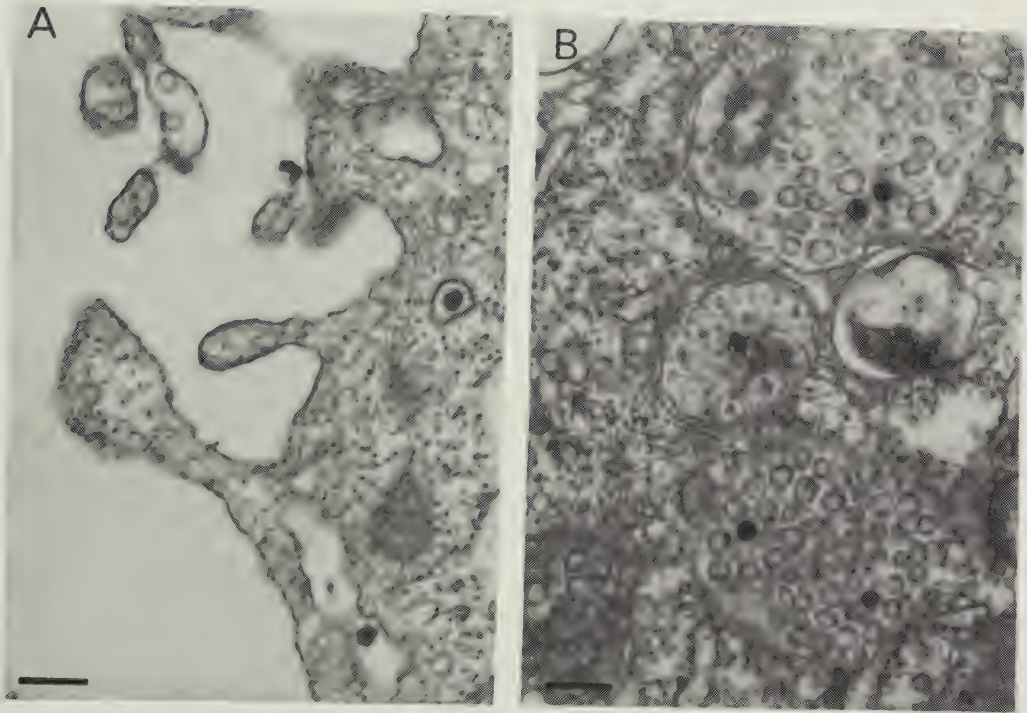


Figure 4

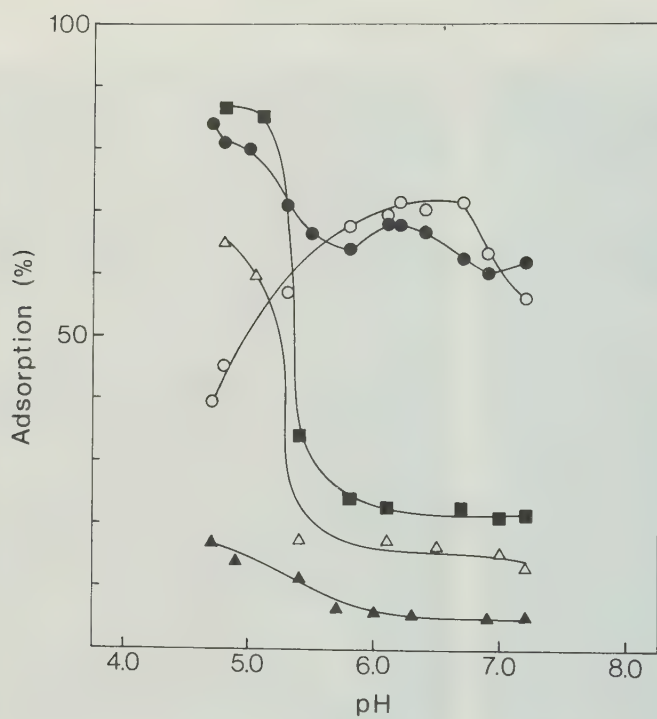


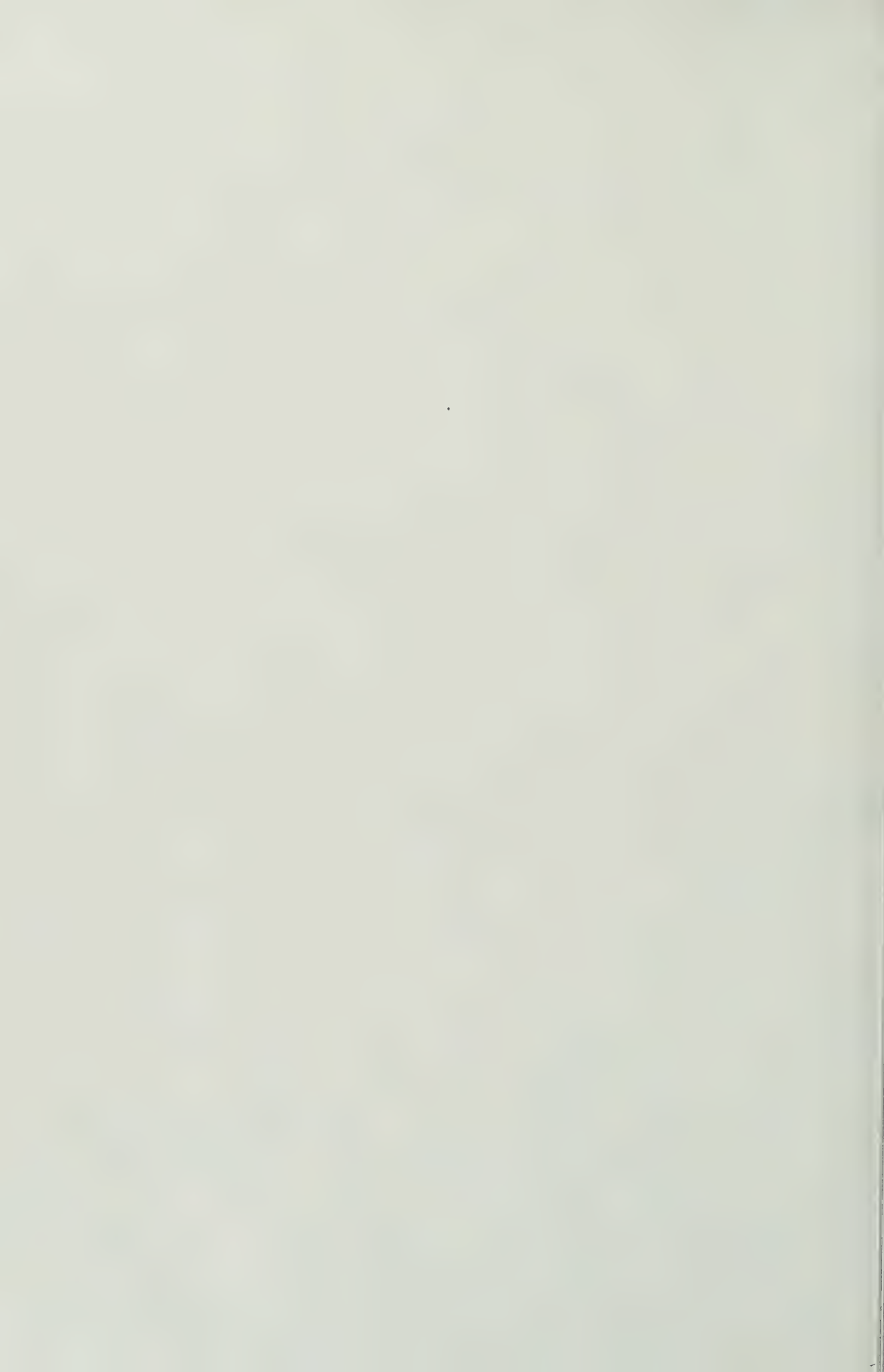
Figure 5



CELLULAR DISTRIBUTION OF AD2 VIRIONS NEUTRALIZED WITH
DIFFERENT ANTISERA.

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ABSTRACT

Three adenovirus 2 (Ad2)-specified immunogens elicited neutralizing antibodies when injected into rabbits; these were the fiber, the hexon and the penton base. Anti-hexon- and antipenton base-neutralized Ad2 virions, attached to HeLa cells to the same extent as untreated control virus and following attachment these viruses also became sensitive to DNase treatment. A fraction of 75-80% of the attached virions penetrated the plasma membrane, which should be compared with an 84-88% penetration level in the control series. A majority of the anti-hexon-neutralized virions were found in intracellular vesicles as revealed in the electron microscope, but in the case of antipenton base neutralization, a maximum of 50% of the virions were retained within vesicles and ca. 30% were free in the cytoplasmic compartment. A value greater than 45% was never obtained for neutralization with a monospecific antipenton base-antiserum, which could imply the existence of alternative pathways for virus penetration into HeLa cells - one of these being sensitive to treatment with antipenton base-antiserum.

Antisera containing antifiber-specificities efficiently precipitated virions and the precipitation data mirrored the degree of neutralization. Antifiber-neutralized virions attached to cells at a 3-5 times greater extent as compared with untreated control virus and the former virions had a reduced ability to become sensitive to DNase treatment. Around 15% of the attached antifiber-treated virions were found as large aggregates inside multivesicular bodies or lysosomes.

INTRODUCTION

Antibody mediated neutralization of viruses has been extensively studied and has been reviewed by Mandel (17,18). In the adenovirus system, the hexon antigen from serotypes 1-5 has been shown to elicit neutralizing antibodies when immunized into different host species (1,8,11,19,21,22,32,35,37,38), although the neutralizing potency of those antisera varies among the serotypes. Antisera against the fiber antigen of serotypes 1,2 and 5 have been reported both to contain (1,19,37) and to lack (11,24,25,35) significant amounts of neutralizing antibodies. These conflicting data could to some extent be explained by the different neutralization assays employed by these investigators. An antifiber-antiserum effectively precipitates virions (4,28) and aggregation of virions *in vivo* should therefore be an obvious way to abolish virus infectivity but this is not always considered as being equivalent with a "true" neutralization (17). Neutralization of adenovirus seems to be largely a type-specific phenomenon (8,19,21,36,37,38,39). Antisera against the penton base and protein IIIa have been reported not to be involved in neutralization of adenovirus type 2 (2,13).

The aim of the present investigation was to establish the role of different viral immunogens in eliciting neutralizing antibodies and to study the subsequent interaction between neutralized virions and HeLa cells. For this purpose, two series of antisera were used. One was a total antiAd2-antiserum from which antifiber- or anti-hexon-antibodies or both were removed, and the other was a series of monospecific antisera produced in response to extensively purified Ad2 structural proteins. Virions neutralized by various antisera were subsequently studied with regard to patterns of attachment, penetration, uncoating and cellular distribution.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were maintained in suspension cultures at densities between 2.5×10^5 to 5×10^5 cells per ml in Eagle minimal essential medium (E-MEM) supplemented with 5% fetal calf serum and 5 μ g per ml of gentamicin. For attachment studies, cells were grown in tissue culture Petri dishes (diam. 60 mm) in Eagle minimal essential medium formulated for monolayer cultures (M-MEM) and supplemented with 10% fetal calf serum and gentamicin as above.

The wild type of human adenovirus 2 (Ad2) was propagated, and purified as previously described (5, 7). ^3H -thymidine labelled virus was prepared according to Svensson et al. (34).

Production of antisera

a) Polyspecific antisera: Antisera against Ad2 were obtained after three successive intramuscular immunizations in rabbits over a four weeks period (9) with 1.4×10^{12} highly purified virions each time, corresponding to 0.39 mg of total protein (14). The immunogens were added equal volumes of Freund's complete adjuvant at the first and second immunization. The rabbits were bled one week after the last immunization and thereafter weekly over a four weeks period. Two separately prepared antisera against intact Ad2 particles were used in this investigation and these will be referred to as no. 111 and 113.

b) Monospecific antisera: The soluble antigens (i.e. hexon, fiber and penton base) remaining after virus isolation by preparative ultracentrifugation on CsCl-gradients were separated by DEAE-cellulose

chromatography essentially as described by Pettersson et al. (23), except that the buffer system was changed to 50 mM Tris-HCl, pH 7.75. The hexon antigen was further purified by QAE-Sephadex chromatography and by negative affinity on a column of antipenton -IgG-Sepharose 4B (see below). The fiber antigen was further purified by CM-Sephadex chromatography and the penton base by QAE-Sephadex chromatography followed by negative affinity on antifiber-IgG- and antihexon-IgG-Sepharose 4B. Purified protein IIIa prepared as described by Everitt and Philipson (6), was obtained from L. Philipson.

Rabbits were immunized with each protein (20-50 µg) according to the schedule above, and preimmune sera were collected before all immunizations. Each monospecific antiserum was titrated by rocket immunoelectrophoresis against the anti Ad2-antiserum no. 113 and subsequently diluted to contain the same concentration of antibody against the appropriate antigen. All antisera were heat-inactivated at 56°C for 30 min before use.

Cyanogen-bromide activation of Sepharose and coupling of antigens

Sepharose 4B in portions of 3 grams was activated by addition of CNBr dissolved in bidistilled water. To the activated gel was added either 14 mg of hexon or 4 mg of fiber antigen, both purified as described above. Incubations and blocking of remaining reactive groups were performed as described by the manufacturer of the Sepharose 4B. The immunoglobulin fractions from monospecific antisera were precipitated in $(\text{NH}_4)_2\text{SO}_4$ at a concentration of 33% relative saturation and were coupled in an analogous way.

Removal of selected antibody specificities from an antiAd2-antiserum

Aliquots of 200 μ l of an antiAd2-antiserum (no. 111) were passed through columns of fiber- or hexon-Sepharose 4B. All UV-absorbing material above 0.01 at 280 nm and not retained by the Sepharose column was collected and dialyzed extensively against bidistilled water. After lyophilization, the absorbed antisera were resuspended in phosphate buffered saline (PBS) to give 4 x the original volume. The specificities of the immunoabsorbed antisera were tested by rocket immunoelectrophoresis (12).

Rocket immunoelectrophoresis (RIE)

Appropriate amounts of antisera, as indicated in Fig. 1, were mixed with 1% agarose in 73 mM Tris-24 mM barbitone buffer, pH 8.6 containing 0.45 mM Ca-lactate. Aliquots of 5 μ l were applied in each sample well and electrophoreses were performed at 75 V, for 16-18 h at 10 $^{\circ}$ C. After electrophoreses, gels were compressed, dried and stained in 0.2% Coomassie brilliant blue R dissolved in methanol-acetic acid-water (45:10:45).

Immunoprecipitation of virions with different antisera

Twentyfive μ l samples of 3 H-thymidine labelled virions (4.4×10^{10} particles) were added an equal volume of the appropriate antiserum, when necessary, diluted in PBS containing 1% BSA to yield final antiserum dilutions ranging from 1/2 to 1/4,000. Higher concentrations of antisera were obtained by adding 100 μ l of the appropriate antiserum to 25 μ l of virus and these series will be referred to as the 1/1.25 samples.

After incubations at 37°C for 30 min, 50 μ l of PBS were added and after thorough mixing the samples were layered onto sucrose gradients formed on top of a cushion of CsCl (1.4 g per ml) and consisting of 2 x 1.5 ml of 10-25% (wt/vol) sucrose in 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.5. Centrifugations were performed at 50,000 x g for 10 min at 5°C. The tubes were fractionated from the bottom into fractions of 5 drops each, and aliquots were assayed for radioactivity in Ready-Solv after precipitation with trichloroacetic acid (TCA) on paper filter discs. For each sample the immunoprecipitated radioactivity recovered on the CsCl-cushion was monitored relative to the rest of the radioactivity of the tube.

Neutralization of virus infectivity measured by progeny virus immunotitration

The neutralization tests were performed according to a progeny virus immunotitration assay as recently described (40). Briefly, 4.4×10^{10} virions in volumes of 25 μ l were added appropriate antisera at final dilutions as described above. After incubations at 37°C for 30 min, PBS containing 1% BSA was added to each sample to give a final volume of 1,550 μ l. Twentyfive μ l samples of antibody-virus complexes were added to 1.52×10^7 cells in 100 ml screw capped Schott Glasswerke Laboratory bottles, yielding a multiplicity of infection (MOI) of 46 physical particles equivalent to one infectious unit per cell (40). After attachment for 30 min at 37°C, 34 ml of E-MEM containing serum were added to each bottle, yielding a concentration of around 4.1×10^5 cells per ml. At 39 h post infection progeny virus was prepared by a one step CsCl-gradient centrifugation procedure. To the virus material was added an equal volume of 10 M urea and progeny virus was quantified

by RIE against an antihexon-antiserum. Neutralized virus samples were compared with separate controls in which the neutralizing antisera were replaced by PBS containing 1% BSA or by a preimmune serum.

In vitro DNase sensitivity of virions after incubation with different antisera

The DNase sensitivity analyzes, both in vitro and on virions attached to cells (see below), were essentially performed as described by Joklik (10). To 4.4×10^{10} ^3H -thymidine labelled virions in volumes of 25 μl were added appropriate antisera at the dilutions described above. After incubations at 37°C for 30 min, the samples were added one volume of 1% BSA in PBS, whereafter 7.5×10^9 particles were withdrawn and incubated for 30 min at 37°C in the presence of 0.2 mg DNase I and 7.5 mM MgCl_2 in PBS, in a final volume of 60 μl . The samples were subsequently diluted with 400 μl of PBS containing 0.1% BSA and undegraded ^3H -DNA was precipitated with TCA at a final concentration of 10%. TCA precipitates were pelleted, washed with ethanol, and dissolved in Protosol. The supernatants and the precipitates were subsequently assayed for radioactivity in 0.4% Omnifluor in toluene-methanol (1:1).

Attachment of virus-antiserum mixtures

Confluent monolayers of HeLa cells were washed with PBS containing 1% BSA. ^3H -thymidine labelled virions were preincubated with the appropriate antiserum for 30 min at 37°C as described above and added to the monolayer cells at MOIs of 2,000 in total volumes of 500 μl of M-MEM. Incubations were performed for 60 min at 37°C on a rocking platform. Unattached virions were aspirated and the cells were washed once with 1% PBS and once with 0.02% EDTA in PBS. The monolayers were incubated

in the presence of the latter solution at 37°C until the cells were released. The radioactivity of the cellular fraction and the washing solutions was measured and the percentage of virus attached was calculated.

DNase sensitivity of virus-antiserum complexes attached to cells

³H-thymidine labelled virions, preincubated with different antisera, were allowed to attach to cells in monolayer cultures for 60 min at 37°C as described above. The cells were released from the Petri-dishes as described above, transferred to centrifuge tubes and sedimented. Resuspension of cells was done in 10 mM sodium phosphate buffer, pH 7.3 containing 10 mM MgCl₂. The cells were disrupted by addition of Na-desoxycholate (DOC) to give a concentration of 0.5%. After addition of 1 mg DNase I and incubation at 37°C for 30 min, the undegraded DNA was precipitated with TCA at a final concentration of 10%. Precipitates were pelleted, washed with 70% ethanol, dissolved in Protosol and assayed for radioactivity.

Electron microscopy (EM)

Cells in suspension cultures were sedimented, washed once and resuspended in PBS at a concentration of 5×10^7 cells per ml. To the cells were added virions, pretreated with the appropriate antisera at concentrations indicated in Table 1, and at calculated MOIs of 5,000. The samples were incubated for 60 min at 37°C, whereafter the cells were washed twice in 10 ml of PBS each time. The cells were resuspended in PBS and kept at 3°C at a density of 1×10^6 cells per ml. Fixation was made in 2% glutaraldehyde for 30 min at 3°C in a roller

bottle, after which the cells were washed three times in PBS. Post-fixation with osmium tetroxide and dehydrations were performed essentially as described by Ryter and Kellenberger (29) and finally the cells were embedded in Epon.

Chemicals

Eagle minimal essential medium for suspension cultures and for monolayer cultures, fetal calf serum and gentamicin were obtained from Flow Laboratories Ltd., Irvine, Scotland. Freund's complete adjuvant was from Difco Laboratories, Detroit, MI. DEAE-cellulose (DE 52) was obtained from Whatman Inc., Clifton, NJ, and Sepharose 4B was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Agarose (type 1) and DNase I were obtained from Sigma Chemical Co., St. Louis, MO. Ready-Solv HP/b was purchased from Beckman Instruments AB, Bromma, Sweden. Omnifluor and Protosol were obtained from New England Nuclear Chemicals GmbH, Dreieich, Fed. Rep. Ger. Na-desoxycholate was from BDH Chemicals Ltd., Poole, England and the Laboratory bottles were from Schott Glaswerke, Fed. Rep. Ger.

RESULTS

Specificity of the immunoabsorbed antisera

Columns of fiber- and hexon-Sepharose 4B were used to selectively remove antifiber-orantihexon-antibodies from an antiAd2-serum (no. 111). The remaining specificities of the antisera were analyzed by RIE against different virion specific antigens (Fig. 1). As compared with the control serum (Panels A and B, Gel 1) it was shown that the antibody titers against the penton base (Panel A) and protein IIIa (Panel B) were not significantly affected by the removal of antihexon-antibodies (Gel 2), antifiber-antibodies (Gel 3) or by both (Gel 4). Moreover, no precipitates against the homologous antigens could be detected among the absorbed antisera when these were tested by RIE against a wide range of concentrations of both antigens and antisera as exemplified in Fig. 1.

Immunoprecipitation and neutralization of virions with different antisera

Virions were mixed with appropriate heat-inactivated antisera as described in Materials and Methods. Such mixtures were both analyzed for possible aggregation by centrifugational rate separation in sucrose-gradients and also assayed for neutralization as described. Two series of antisera were used. One was a total antiAd2-serum (no. 111) together with the absorbed antisera derived from the same serum pool (described above) and the other was a series of monospecific antisera, titrated in RIE and diluted to contain the same amount of each antibody specificity as a reference antiAd2-serum (no. 113).

As shown in Fig. 2, the precipitation patterns (solid bars) mirror the neutralization data (open bars), i.e. a high percentage of precipitation yields a correspondingly high degree of neutralization. The most striking effects were observed with antisera containing anti-fiber-specificities, i.e. the two antiAd2-sera (no. 111 and 113), the total antiAd2-serum (no. 111) minus antihexon-antibodies and the monospecific antifiber-serum. For these four antisera a final dilution of 1/200 displayed an almost 100% neutralization and a 100% precipitation (Fig. 2). The total antiAd2-serum minus antifiber-antibodies revealed a close to 100% neutralization at a final serum dilution of 1/8 and the antihexon-serum revealed an 80% neutralization and a 30% precipitation at a final dilution of 1/2. When both antifiber- and antihexon-antibodies were removed from the total antiAd2-serum, a neutralization of around 85% was observed at a final dilution of 1/1.25. This effect was probably due to the antipenton base-antibodies, since a monospecific antipenton base-serum displayed a 45% neutralization at the same final dilution. A notable feature of the neutralization tests with the antipenton base-serum was the fact that no further increase in neutralization was achieved at higher serum concentrations; thus a final serum dilution of 1/1.1 gave the same result at a 1/1.25 dilution (not shown). Preimmune sera and an anti-protein IIIa-serum were both totally devoid of antibodies causing precipitation or neutralization.

In summary: Three classes of antibodies showed inhibitory effects on virus infectivity, these were antifiber-, antihexon- and antipenton base-antibodies.

Antibody-mediated destabilization of virions in vitro

The possibility that incubations of Ad2 with various antisera could destabilize the virions in vitro, thus rendering the genome sensitive to DNase treatment was investigated. Virions were mixed with the appropriate antisera at the same final concentrations as described above and samples were subsequently analyzed for DNase sensitivity. After virus treatment with antisera containing antifiber-specificities, i.e. total antiAd2-serum, antiAd2-serum minus antihexon-antibodies and antifiber-serum, 15-20% of the labelled virion material became sensitive to DNase treatment at final serum dilutions of 1/8 (not shown). A less pronounced tendency of destabilization was observed at both higher and lower dilutions of all antifiber-containing antisera. These observations could reflect the existence of a critical ratio between antifiber-antibodies and virions. No other antisera nor the pre-immune serum displayed any effect on viral DNase sensitivity.

Attachment of virus-antibody complexes to HeLa cells

Attachment of the total mixtures of virus particles incubated with appropriate antisera were analyzed on monolayer cells and compared with a control in which virus was preincubated with either 1% BSA in PBS or with a preimmune serum. When virions were treated with any of the antifiber-containing antisera at dilutions ranging from 1/8 to 1/1,000, an increase was observed in virion attachment by a factor of 3 to 5 as compared with the control (Fig. 3). The amount of virions attached in the presence of those antisera, decreased when the serum concentrations were lowered from 1/1,000 to 1/4,000 (Fig. 3).

The attachment of virions preincubated with antihexon-serum, anti-penton base-serum or antiAd2-serum minus both antifiber- and antihexon-antibodies did not significantly differ from the results obtained after incubations with a preimmune serum (Fig. 3). The antiAd2-serum minus antifiber-antibodies did not influence the attachment, except at the highest serum concentration.

DNase sensitivity of virus-antibody complexes attached to cells

In a previous report it was suggested that virion destabilization takes place already at the cell surface (33). To examine whether pre-treatment of virions with various antibodies in any way affected the normal uncoating, virus incubated with various antisera were allowed to attach to monolayer cells, and attached virions were subjected to DNase treatment as described in Materials and Methods. The back-ground level of DNase sensitivity in vitro, as discussed above, was subtracted from the values obtained after the interaction of virus-antibody complexes with cells. All antifiber-containing antisera displayed a pronounced inhibitory effect on the cell-mediated destabilization of the virus particles (Fig. 4), whereas antihexon- or antipenton base-neutralized virions became destabilized to the same extent as the untreated control. However, virions pretreated with antiAd2-serum minus antifiber-antibodies at a reciprocal serum dilution of 1.5 were not destabilized to the same extent as the control.

Fate of antibody treated virions attached to cells

In analogy with a previous study on virion internalization (33), ^{125}I -labelled anti-rabbit-immunoglobulin G was used to quantify the degree of internalization of attached virion-antibody complexes. In

the presence of either antihexon- or antipenton base-sera (dil. 1/1.25), ca 85% of the virions were internalized. This figure was reduced by half when an antifiber-serum (dil. 1/200) was tested (not shown). To further follow the cellular distribution of virions treated with various anti-sera, an electron microscopic investigation was performed (Table 1). After treatment of virions with a total antiAd2-serum or with an antifiber-serum, ca. 85% of the virions remained on the surface. The fifteen per cent of the virions which were internalized appeared in large aggregates and were located in vesicular structures such as multivesicular bodies or lysosomes (Fig. 5A). Virions preincubated with an antihexon-serum or an antipenton base-serum entered the cells nearly as efficiently as virions treated with a preimmune serum (Table 1). In the case of antihexon-serum, ca 70% of the virions were accumulated inside vesicles and only 8% were found near the nucleus. After pretreatment with antipenton base-serum, around 50% of the virions were left inside vesicles, which usually contained only a few virus particles, as also was the case with antihexon-treated virions (Fig. 5B and 5C). However, 25% of the antipenton base-treated virions appeared near the nuclear membrane. In the control series ca. 55% of the virions reached the nucleus (Table 1 and Fig. 5D).

DISCUSSION

In this communication we have demonstrated that three different viral immunogens elicit neutralizing antibodies as judged by reduction in progeny virus yield. A monospecific antifiber-serum displayed a neutralizing capacity of the same magnitude as a total antiAd2-serum. When the antifiber-antibodies were removed from an antiAd2-serum, the remaining neutralizing potential was of the same order as an antihexon-serum, when the antiAd2-serum was depleted of both antifiber- and antihexon-antibodies the residual neutralizing activity equalled that of an antipenton base-serum.

A notable feature of the neutralization assays was the observation, especially among the antifiber-containing antisera, that the extent of neutralization paralleled the precipitation patterns. This raises the question whether the present neutralization data simply reflect the degrees of aggregation of virions, i.e. aggregation of several infectious units into a single infectious entity, which subsequently leads to a lower number of cells being infected than expected in the immunotitration assay. If this was true, addition of more than one infectious unit per cell would increase the total virus yield in the immunotitration assays. We have tried this hypothesis among the used antisera by adding up to 250 neutralized infectious units per cell, but no increase in virus yield was observed.

Despite aggregation of virions with antifiber-containing antisera, such complexes had a capacity to attach to cells at an even greater extent than untreated virions. An unaltered or enhanced attachment efficiency after treatment with neutralizing antisera has been described for adenovirus (26) and in other virus systems e.g. poliovirus (15) and influenza virus (27). The attached Ad2

virions treated with antiAd2- or antifiber-serum did not become sensitive to DNase treatment to the same extent as the controls and these virions remained on the cell surface (85%) or were located inside vesicles (15%). This cellular localization is in good agreement with the observations obtained by Brown and Burlingham (3) who used KB-cells and an antiAd2-serum. From the results above it is possible to suggest that the reduction in virus yield in the immunotitration assays when virions were incubated with antifiber-containing antisera, is not just a reflection of aggregation, but is also dependent on a neutralizing mechanism apart from a mere precipitation.

When the data from antihexon- and antipenton base-neutralization were analysed it was found that the percentage of neutralization was always "higher" than the percentage of virion precipitated. Virions incubated with either of these antisera attached to cells and became sensitive to DNase treatment to the same extent as the control. It was previously shown that glutaraldehyde-stabilized virions became internalized but were unable to leave an endocytotic vesicle (U. Svensson, submitted to J. Virol.). When antihexon- and antipenton base-sera were used at neutralizing concentrations, the majority of the virions were internalized but confined within vesicles. In the poliovirus and influenza virus systems, it has been demonstrated that neutralized virions not only attach to cells but also penetrates the cell membrane and influenza virus is uncoated as well (16,27). The interaction between the penton base and the endosome membrane has been suggested to be important during adenovirus entry from an acidic vesicle into the cytoplasmic compartment (30,31,U. Svensson, submitted to J. Virol.). Possibly this interaction was blocked as a result of the reaction between virus and antipenton base-antibodies.

However, 25% of the virions incubated with this antiserum succeeded in reaching the cell nucleus. This agrees well with the result that an antipenton base-serum never neutralized adenovirus by more than 45%. Those facts also supports the earlier proposal that not all virions are dependent on an acidic vesicle and the penton base to enter into the cytoplasmic compartment (U. Svensson, submitted to J. Virol.). This entry pathway is at present uncharacterized, however, an alternative entry mechanism, direct penetration, has earlier been described (3,20).

It is obvious that when the three monospecific neutralizing antisera were used, the cellular distribution of neutralized virions was not the same, thus an antifiber-serum generally inhibited the virus internalizing and the antihexon- and antipenton base-sera prevented the entry of virions from acidic vesicles into the cytoplasmic compartment.

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Table 1

Cellular distribution of Ad2 particles after incubation with various antisera

Treatment	Relative distribution of Ad2 particles (%)			
	Cell surface	Vesicles	Free in the cytoplasm	Free near the nucleus
AntiAd2-serum no. 113, (1/200) ^a	88	12	0	0
Antifiber-serum (1/200)	85	15	0	0
Antihexon-serum (1/1.25)	21	69	2	8
Antipenton base-serum (1/1.25)	25	47	3	25
Preimmune serum (1/1.25)	16	16	12	56
PBS	12	14	20	54

^a The figures within parentheses refer to the final dilutions of antiserum in the reaction mixture.

FIGURE LEGENDS

Figure 1

RIE showing the specificities of the immunoabsorbed antisera.

The agarose gels contained the following antisera:

- 1) AntiAd2-serum (no. 111)
- 2) AntiAd2-serum minus antihexon-antibodies
- 3) AntiAd2-serum minus antifiber-antibodies
- 4) AntiAd2-serum minus antihexon- and antifiber-antibodies

Panel A: the gels contained 0.5% of each antiserum.

f = fiber antigen, h = hexon antigen and pb = penton base antigen

Panel B = the gels contained 2% of each antiserum.

Protein IIIa was applied in all wells.

Figure 2

Neutralization and precipitation of virions by different antisera.

Ad2 virions were mixed with different antisera as indicated in the figure and the mixtures were analyzed for aggregation in sucrose-gradients (solid bars) and for neutralization by the progeny virus immunotitration method (open bars).

^a The figures refer to the percentage of reduction in progeny virus yield as compared with the untreated control and to the percentage of radioactivity recovered on a cushion of CsCl in sucrose-gradients, for the neutralization and precipitation studies, respectively.

* In this precipitation sample, the reciprocal serum dilution was 1.5.

Figure 3

Attachment of Ad2 virion-antibody complexes to cells. ^3H -thymidine labelled virions were preincubated with different antisera and allowed to attach to monolayer cells for 60 min at 37°C . Cells were harvested and the relative amount of attached virions was calculated after radioactivity measurement as described in the text.

^a The figures refer to the percentage of attached virions.

* In this sample the reciprocal serum dilution was 1.5.

Figure 4

DNase sensitivity of virion-antibody complexes attached to cells. ^3H -thymidine labelled virions treated with different antisera were attached to monolayer cells for 60 min at 37°C . The cells were harvested and the DNase sensitivity of the parental virion DNA was estimated as described in the text.

^a The figures refer to the relative DNase sensitivity of the viral genome compared with the uncoating efficiency of untreated control virions.

* In those samples the reciprocal serum dilution was 1.5.

Figure 5

Cellular distribution of antibody-treated adenovirus as observed in EM. Virions, pretreated with different antisera, were incubated with cells for 60 min at 37°C . Panels show cells with virions pre-treated with; (A) antifiber-serum, dilution 1/200; (B) antihexon-serum, dilution 1/1.25; (C) antipenton base-serum, dilution 1/1.25; (D) preimmune serum, dilution 1/1.25. Bar, 200 nm.

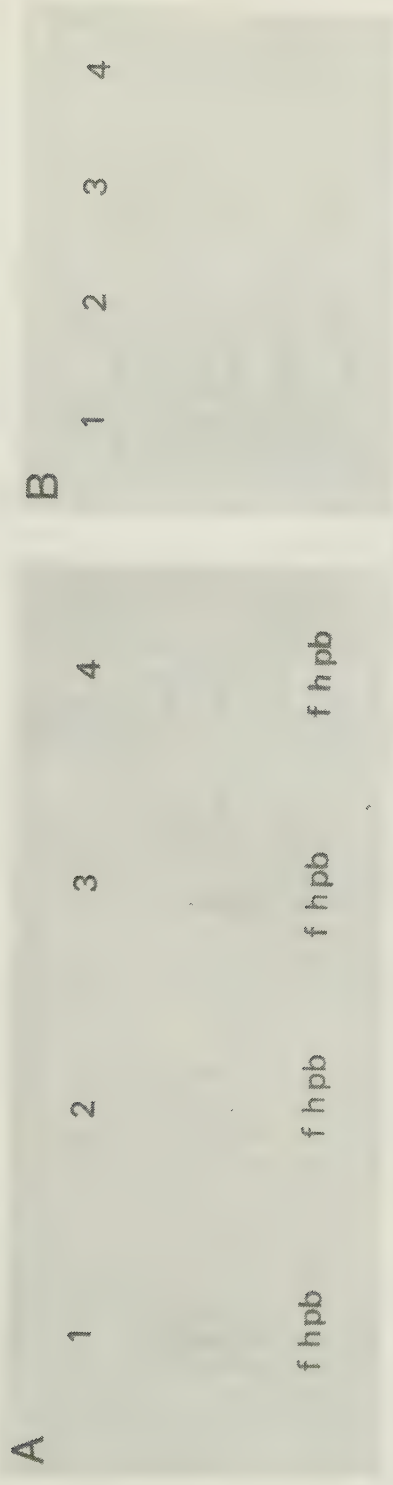


Figure 1

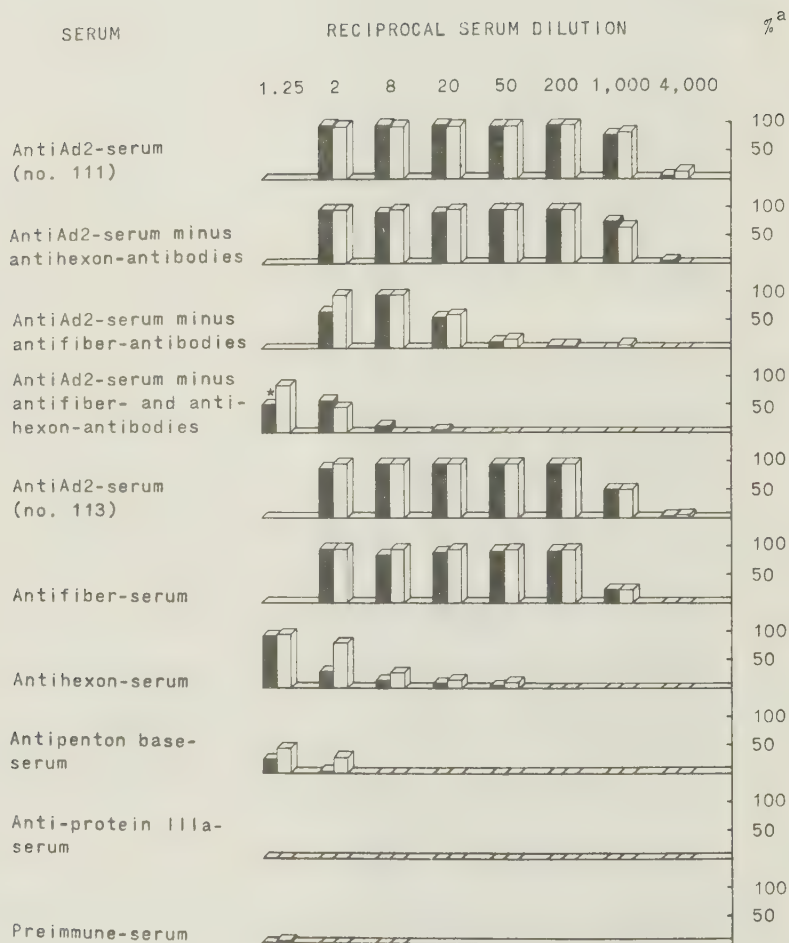


Figure 2

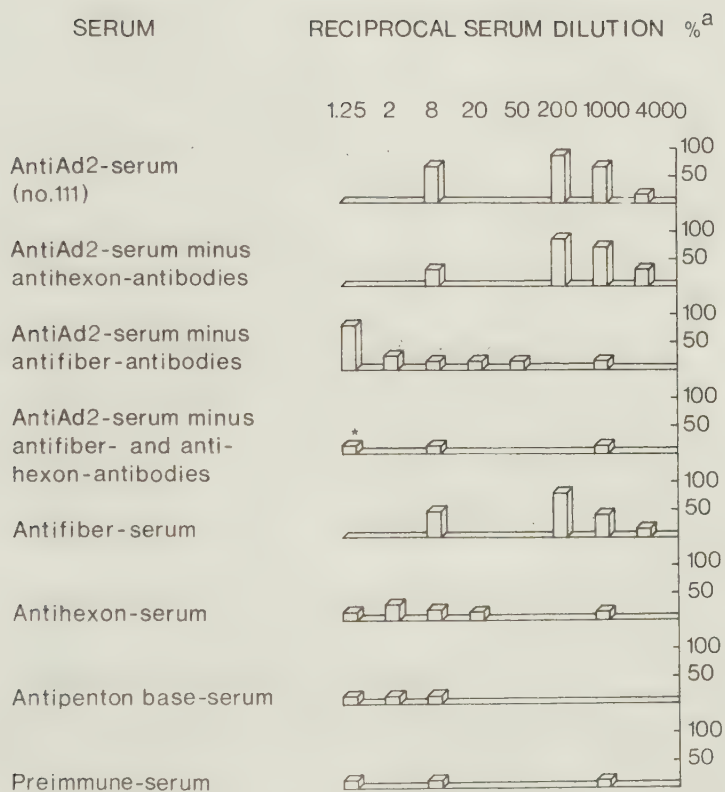


Figure 3

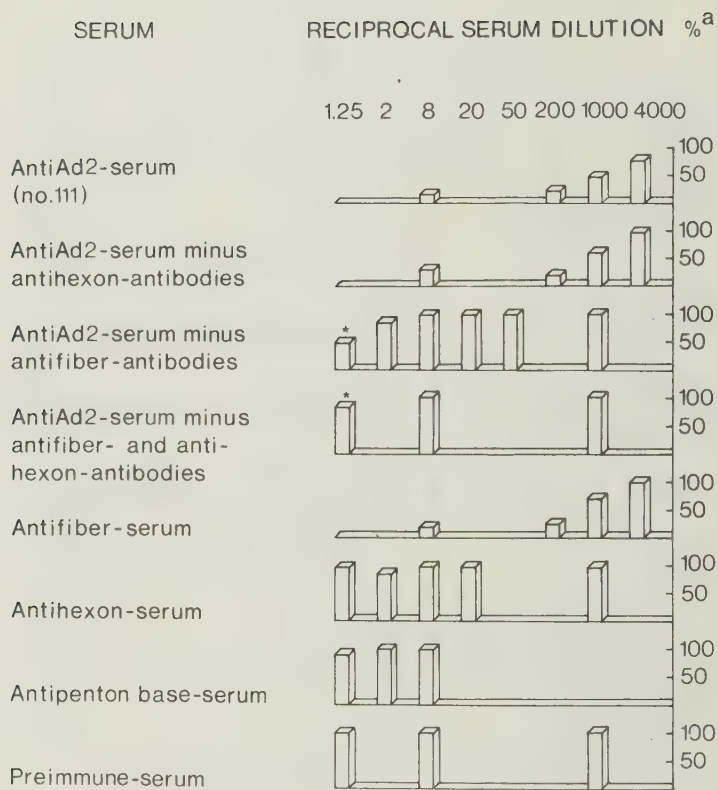


Figure 4

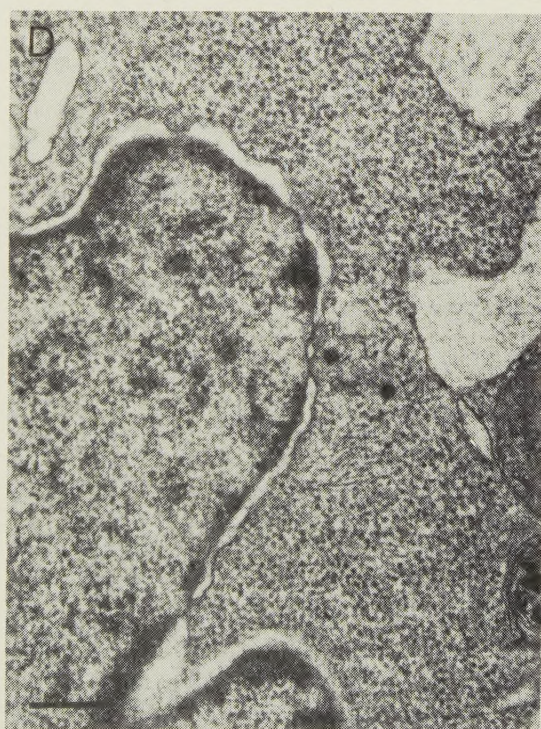
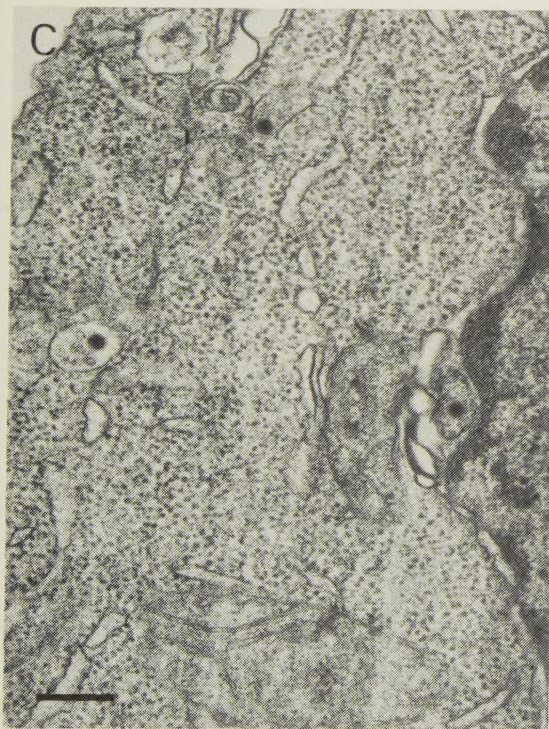
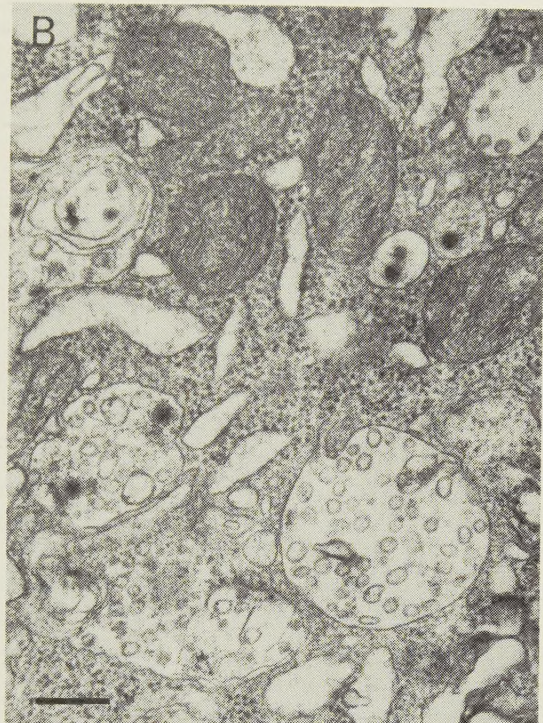
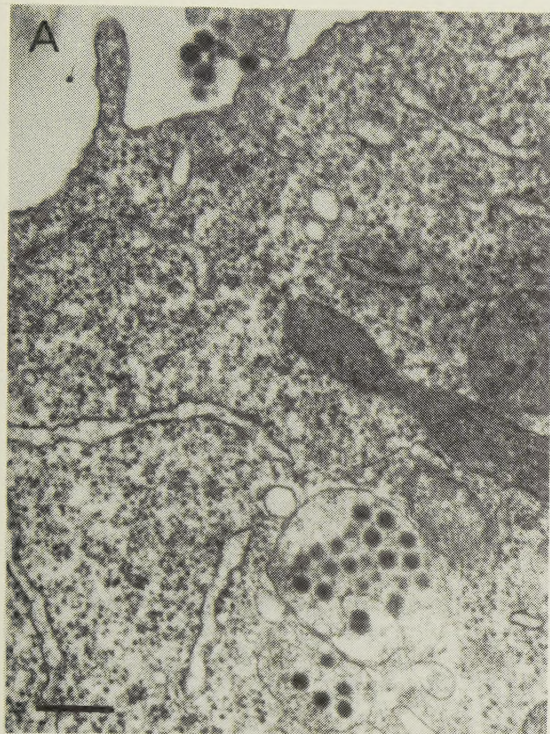


Figure 5

